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The cell cycle is regulated by the action of a family of cyclin dependent kinases (Cdks) which catalyze particular cell cycle transitions. Cdks are positively regulated through interaction with cyclins and are negatively regulated through phosphorylation and through association with inhibitory proteins of the CIP/KIP and INK4 families. Our research has focused on the role of p57KIP2 in development and cancer. We have found that p57KIP2 is expressed in a highly cell type specific manner during embryonic development and in adult tissues being most highly expressed in terminally differentiated cells. Through analysis of p57KIP2 deficient mice, however, we have found that p57KIP2 is required for normal development of several tissues including kidney, lends, muscle, and bone. We also discovered the p57 is involved in the human overgrowth and cancer predisposition disease Beckwith-Weidemann Syndrome, BWS. Current studies are aimed at understanding in greater detail the function of p57KIP2 and its possible role in human cancers.

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Introduction

The eukaryotic cell cycle is regulated primarily at two points, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The commitment to a round of cell division is made at a point in G1, referred to as the restriction point in mammalian cells[1-4] or START in yeast[5]. Passage through the restriction point depends critically on mitogen signals, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner[4]. Passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

Cyclins, Cdks, and positive growth control. Cdks are protein kinases that require association with cyclins and phosphorylation for activity[5-8]. Cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks[5-12]. Cyclins D and E function in G1[6, 10, 13-16], and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase[1, 3, 17, 18]. Amplification of cyclins, D1, D2 and E have been identified in several tumors[19-23]. Cyclin D1 was identified as the PRAD1 oncogene [24]. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma[25]. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

Cyclins D1,D2, and D3 bind Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb[6, 26-29]. Because D-type cyclins are required for proliferation only if cells have an intact Rb gene, it is thought that Rb inactivation is their primary role. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition[14, 15, 30-33]. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition[31]. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition [34-36], while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Although Cdks are thought to be the critical regulators of cell proliferation, little is known about how cyclin/Cdk complexes regulate cell proliferation during development. In this regard, we have performed an analysis of the expression of the major cyclins during mouse embryonic development and in adult tissues. We have discovered that general cyclins E, A, B, and F are expressed in all proliferating tissues while the D cyclins are distributed in a pattern distinct for each cyclin but which is a subset of the general cyclins (Parker, Harper and Elledge, unpublished results). This is consistent with the notion that D cyclins are the primary initiators of cell cycle entry and orchestrate development. We have recently observed that cyclin D1 is the only D-type cyclin induced when breast cells proliferate during pregnancy. We and our collaborators in the Weinberg laboratory discovered that development of the breast during pregnancy is dependent upon cyclin D1[37]. As mentioned above, amplification of D-type cyclins is frequently observed in breast cancer. This provides a link between development and cancer and indicates that the developmental history of the breast is relevant to its susceptibility to tumorigenisis.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. Another example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development [37]. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding

of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

Tumor suppressor proteins and negative growth control. Rb and p53[29, 38] are the most well understood tumor suppressors. Mutations in these are found frequently in many human cancers[39,40], and reintroduction of wild-type genes into $p53^-$ or Rb^- tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis[28, 41, 42].

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors[39] including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included[43]. p53 deficient mice are prone to the spontaneous development of a variety of tumor types[44]. Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53[45-53]. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification[54, 55]. p53 regulates the expression of $p21^{CIP1}$, an inhibitor of G1-cyclin/Cdks, in response to DNA damage[56-60]. Using a p21 knockout mouse, we have determined that p21 is required for full function of the G1 checkpoint in response to γ-irradiation, although there is residual checkpoint function[56]. Furthermore, these mice do not show the high rate of spontaneous tumor formation seen in p53-deficient mice. It is not clear whether p53's role in oncogenesis is through its checkpoint or apoptotic deficiencies, or a combination of these.

The current view of the role of Rb in the cell ccyle is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase[29]. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

The above observations are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

The cell cycle and development: potential roles for Cdk inhibitors. Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. While much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the non-proliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a non-proliferative state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. While the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdks is likely to be a central feature. Recently a new class of Cdk regulatory molecules have emerged that are potential mediators of cell cycle exit and maintenance of the non-proliferative state. These are the inhibitors of cyclin-dependent kinases, CKIs. Currently two

structurally defined classes of CKIs exist in mammals that are exemplified by $p21^{CIP1}$ (57-60) and $p16^{INK4/MTS1}$ (61-65).

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. Cdk inhibitory proteins are a group of proteins that associate with and inhibit Cdks. These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors $p21^{CIP1/WAF1}$ (57-60) and $p16^{INK4a}$ (61) had only recently been identified. Subsequently, we and others identified additional inhibitors including p27, p57, p15, p18, and p19 (refs 61-69). We identified $p21^{CIP1}$ in a two-hybrid screen designed to identify proteins that associate with Cdk2 (57). Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory (59), as a Cdk associated protein by the Beach laboratory (58), and as an S-phase inhibitory cDNA in senescent cells (60). Since then we and others have identified two other members of the p21 family, p27 and p57. p57, also known as KIP2 has been the focus of this study. It is expressed in the breast and is localized to 11p15.5m a locus involved in breast cancer (see below).

Involvement of 11p15.5, the location of KIP2, in human cancers including cancer of the breast. *Several chromosomal regions show frequent loss of heterozygosity(LOH) in breast tumors including but not exclusively 3p, 7q31, 11p15, 11q13 and 17p(reviewed in 96). The chromosomal location of KIP2, 11p15.5, marks it as a candidate tumor suppressor gene of the breast. The involvement of 11p15 in the breast is well documented(113-119). 35% of breast tumors show LOH at 11p15.5(119) and this LOH is associated with poor prognosis(119). Furthermore, 11p15 LOH has been associated with metastasis(116) and there is evidence that 2 distinct breast tumor suppressor genes may reside at this locus(118).* 11p15 has also been intensively investigated because of frequent LOH at this locus in a number of other human cancers including bladder, lung, ovarian, kidney, and testicular carcinomas (reviewed in 70). Several childhood tumors including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma show specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have also indicated a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma (reviewed in 71), either of which could be due to loss of a Cdk inhibitor. In addition, rearrangements in the 11p15 region are found in Beckwith-Wiedemann Syndrome (BWS) which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegely (enlarged organs) and an increased risk (7.5%) of childhood tumors (72,73). Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting(reviewed in 74). Several features of KIP2 make it a reasonable candidate as a mediator of some phenotypes of BWS. First, a Cdk inhibitor could explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS including the tongue, kidney, muscle, and the eye. Third, KIP2 is imprinted and maternally expressed. Furthermore, LOH at 11p15 in Wilms' tumors are exclusively maternal, offering further support for the possibility that KIP2 might be the WT2 gene. LOH of the breast has not yet been examined for parental specificity of LOH. However, the potential for the existence of two tightly linked tumor suppressors(75) affecting the breast at 11p15 might complicate the analysis of parentally biased LOH depending on the relative frequency of the two events. Nevertheless, the biochemical properties of KIP2, its physical location and expression patterns suggest that it may be the tumor suppressor at 11p15.

A role for p57 in Beckwith-Wiedemann Syndrome.

As mentioned above, Beckwith-Wiedemann Syndrome (BWS) is a clinically variable disorder characterized by somatic overgrowth, macroglossia, abdominal wall defects and visceromegaly (83). Children with BWS are also susceptible to a variety of childhood tumors, including Wilms' tumor, hepatocellular carcinoma and rhabdomyosarcomas. The disease, which affects 1 out of every 13,700 live births each year, is genetically heterogeneous, with the majority of cases occurring sporadically. Familial cases, which represent ~15% of BWS patients, have helped establish a genetic linkage of BWS to human 11p15.5, where there is a large cluster of imprinted genes (84, 85). That defects in imprinted genes might explain the etiology of the syndrome was first suggested from observations that both familial and sporadic cases can be associated with 11p15.5 partial paternal uniparental disomies (UPDs) and trisomies with paternal duplications (86, 87).

Many of the key defects found in BWS patients could be explained by alterations in the control of cell proliferation, either in the context of organogenesis or tumorigenesis. Thus, candidate genes should be tied in some respect to the control of cell proliferation as well as being imprinted. Two candidate imprinted genes with these properties map to 11p15.5, *IGF2* and $p57^{KIP2}$. These genes have strikingly similar patterns of expression during development in mice and are expressed in all of the tissues affected by BWS (88, 89, 90).

IGF2 encodes a fetal-specific growth factor that is paternally expressed in both mice and humans (91, 92, 93). When the expression of *IGF2* was examined in BWS patients, it was shown to be elevated as the result of deregulation of its imprinting in some, but not all cases with normal karyotypes (94, 95). Furthermore, paternal trisomies and UPDs would be expected to double the expression of *IGF2* and could potentially account for overgrowth observed in these cases of BWS.

Maternally inherited loss-of-function mutations in the $p57^{KIP2}$ gene have also been identified in ~5-15% BWS cases examined (96, 97) and 30%-50% of familial cases cases (98). $p57^{KIP2}$encodes a member of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs), and is maternally expressed in all mammals examined to date (99,89,90,100,101). CKIs of this class inhibit G1/S phase cyclins, and the absence of $p57^{KIP2}$ has been shown to affect the ability of cells to exit from the cell cycle. The gene lies ~800 kb from *IGF2* in both mouse and human (102, 103, 104).

A small number of patients (<1%) have been identified with balanced translocations or inversions 3' of $p57^{KIP2}$ in a neighboring imprinted gene, *KvLQT1* (105, 106, 107). This gene encodes a voltage-gated potassium channel that is maternally expressed in humans in all tissues except the heart, but mutations in the gene have been implicated only in the cardiac arhythmia long QT syndrome, not BWS (108, 102). It has been suggested that the regulation of other genes in the locus, particularly $p57^{KIP2}$and *IGF2*, may be disrupted by the translocations. Indeed, in one such family, *IGF2* expression has been shown to be biallelic (109). Finally, the methylation imprint and expression of a recently described paternally expressed transcript within an intron of *KvLQT1, LIT1*, has been shown to be disrupted in over 50% of BWS patients examined (110, 111, 112). These groups suggest that this transcript may mediate the imprinting of other genes in the locus, although they disagree on the likeliest candidates (110, 111, 112). Several mouse models that shed light on the etiology of BWS have been generated. These studies have confirmed that embryonic growth in mice is very sensitive to the levels of the growth factor IGF2. When a mutated copy of the *Igf2* gene was inherited paternally, the offspring were 60% the size of their wild-type littermates (91). In contrast, in mice in which the cis-acting sequences that control *Igf2* imprinting were deleted (*H19-13*), *Igf2* was expressed from both parental chromosomes, and the offspring displayed somatic overgrowth and placentomegaly, but none of the other symptoms of BWS (113, 114). H19 is a gene that encodes and RNA of unknown function. Its significance to IGF2 is that it is oppositely imprinted and that deletion of DNA in the H19 gene disrupts IGF2 imprinting and allows IGF2 to be expressed off of both maternal and paternal chromosomes. This mutation raised the tissue levels of IGF2 2-fold, but had a less pronounced effect on its circulating levels.

Mutations in the type 2 *Igf2* receptor gene (*Igf2r*), whose product binds *IGF2* and targets it for lysosomal degradation also caused elevation of IGF2 (115, 116, 117). Circulating IGF2 levels were

elevated ~4-fold, and embryos died late in gestation with many, but not all, of the phenotypes of BWS, such as somatic overgrowth, placentomegaly, heart hypertrophy, omphalocele and adrenal cysts. In *Igf2r* and *H19-13* double mutants (114), IGF2 levels were increased 7-11 fold, and the severity of the phenotypes were more pronounced than in either single mutant. In these mice, however, the macroglossia, renal dysplasia and adrenal cytomegaly commonly found in BWS patients were missing. Overexpression of IGF2 has also been achieved in mice carrying *Igf2* transgenes (118). These animals exhibited overgrowth, polydactyly and polyhydramnious, all symptoms of BWS patients. Together, these animal models support the hypothesis that BWS results from elevated expression of *IGF2*.

The impact of loss-of-function mutations in $p57^{Kip2}$ has also been examined in mice (119, 120). These mice show abdominal wall defects reminiscent of those seen in *Igf2r/H19* double mutants. They also display a unique set of defects such as renal dysplasia and adrenal cytomegaly, defects seen in BWS patients but not observed in mice with elevated IGF2 expression. The somatic overgrowth commonly associated with BWS, however, was not observed. In addition, phenotypes not previously associated with BWS, such as lens and gastrointestinal tract abnormalities as well as skeletal defects were present in $p57^{Kip2}$-null mice.

These mouse models have failed to provide a good explanation for the fact that BWS patients with loss of imprinting of *IGF2* and those with mutations in $p57^{KIP2}$ are phenotypically indistinguishable. Zhang et al. (120) suggested that IGF2 and p57 may act in opposing manners to control cell proliferation during development of human fetuses; that is, that a gain of function of *IGF2* may act similarly to a loss of function of $p57^{KIP2}$. We reasoned that if IGF2 and p57 act antagonistically during development, a double mutant in which both BWS-potentiating mutations are present might exhibit phenotypes that are more severe than the sum of those in the single mutants. Such a mouse strain would mimic patients with UPD of 11p15.5, with respect to *IGF2* and $p57^{KIP2}$. To generate such a mouse we bred a loss-of-function $p57^{Kip2}$ mutation to an *Igf2* loss-of-imprinting mutation (*H19_13*) and screened for meiotic recombination between these tightly linked genes in the next generation. The double mutant mice exhibit aspects of BWS that have not been observed in other mouse models, such as macroglossia. In addition, they show an exacerbation of the placental and kidney dysplasias caused by the $p57^{Kip2}$ mutation alone. Significantly, we observed that a null mutation in *Igf2* can overcompensate for these severe placental and kidney dysplasias, leading us to suggest that the two genes act in an antagonistic manner in some tissues in the mouse.

The goals of our work were: 1) to determine whether p57 is imprinted in the breast, 2) to construct mice lacking p57, 3) to analyze the phenotype of mice lacking p57, 4) to analyze the role of the QT domain in p57 function by looking for binding proteins, and 5) to characterize the regulation of p57 and 6) to look for additional CKIs in the breast. To date, we have made significant progress on these initial goals. Our progress in these areas is summarized below.

Body

Aim 1: Determination of p57 imprinting status in the breast.

We have completed this Aim and described this in a report two years ago. We found that KIP2 was imprinted in all tissues and this was published in (1996 *Proc. Natl. Acad. Sci. USA* . 93: 3026-3030) and in (1997, *Nature* 387:151-158). All tissues in the $p57^{-/+}$ heterozygous mouse were examined and none were found to express p57 if the null allele was inherited from the mother. A variety of fetal human tissues were examined using a polymorphism and imprinting was found in all tissues examined. The papers are now attached as requested.

With respect to the role of p57 in breast cancer, it is unlikely that it is a causal factor alone because individuals who have no p57, BWS patients, do not have elevated breast cancer rates. It may contribute in some capacity in cases where its imprinting is altered as other genes in the 11p15 region are suspected to have roles, genes such as IGF2. However, if p57 imprinting is altered in those cases, which has not been shown, then it is unclear if it actually is contributing to the tumorigenesis.

Aim 2: Construction of mice deficient in KIP2. We completed this aim and this was published in (1997 *Nature* 387:151-158) which is now attached. Basically we used standard gene replacement technology to delete the p57 gene in ES cells and replace it with the Neo gene. These ES c ells were then introduced into blastocycts and chimaeric mice were generated and bred to allow germline transmission. Pups that had inherited the p57 gene distruption were identified by genomic southern analysis and used to analyze the phenotypes.

Aim 3: Analysis of p57 mutants animals.

We have completed an exhaustive analysis of the mutant phenotypes present in the p57 mutant and p57/27 double and p57/p21 mutant animals in last two years and discovered multiple roles for these proteins in eye, lung, and muscle development. This was published in (1997, *Nature* 387:151-158.) and in (1998 Genes and Dev. 12: 3162-3167.) and (1999 *Genes and Dev.* 13: 213-224). In the last year we have investigated the possible overlap between p57 loss and increases IGF2 growth factor expression in mouse models for the cancer syndrome BWS by making mice mutant for both p57 and H19, a negative regulator of IGF2 imprinting. H19 mutations express 2-fold more IGF2 and IGF2 overexpression has been implicated in BWS. The phenotypes of mice lacking these two genes are discussed below in the order in which we detected them. This was recently published in (2000 *Genes Dev.* 13:3115-24).

A. Generating a $p57^{Kip2}$/H19 double mutant

The phenotypic consequences of mutations in $p57^{Kip2}$ and *H19* are manifested only when the genes are inherited from mothers. Because these genes lie ~900 kb apart in the mouse, a double mutant strain could be generated by meiotic recombination between the existing mutations. Towards that end, we crossed heterozygous $p57^{Kip2+/-}$; $H19\Delta13^{-/+}$ males to C57BL/6 females and screened progeny for animals that were heterozygous for both $p57^{Kip2}$ and $H19\Delta13$ (*p57H19).* Among 481 offspring, we identified two *p57H19* recombinants, a frequency consistent with the estimate of the genetic distance between the genes (0.5 cM). One recombinant, a male, was fully viable and fertile and was used to establish the line. The other recombinant, a female, failed to give birth to viable *p57H19* mice.

Loss of *Igf2* imprinting, and the absence of maternal $p57^{Kip2}$ and *H19* expression were confirmed by RNAse protection and RT-PCR assays. The imprinting and expression of *Kvlqt1* was unchanged (data not shown).

B. Perinatal Lethality

Maternal inheritance of a null allele of $p57^{Kip2}$ is lethal, with 10% of offspring dying *in utero* and the remainder dying within the first two weeks after birth (120). We were unable to recover live *p57H19* mice at birth, suggesting that the double mutant phenotype is more severe. Furthermore parturition invariably occurred at 19 d.p.c., at least one day earlier than in $p57^{Kip2}$ single mutants. At 18.5 d.p.c., *p57H19* embryos were present at approximately the expected frequency (Table 1), suggesting that death occurred during or shortly after delivery.

C, Prenatal Growth

One of the characteristics of BWS is prenatal somatic overgrowth. It had been shown previously that offspring inheriting the H19_13 mutation maternally are born 30% bigger than their wild-type littermates (113, 114). $p57^{Kip2}$ mutants, on the other hand, display no somatic overgrowth at birth (119, 120). Although *p57H19* embryos were indistinguishable in weight from wild-type littermates at e18.5, they were ~20% larger at e16.5-17.5 just as was seen in the single *H19Δ13* deletion at the same time (113). It is conceivable that the gain in growth rate mediated by the loss of *Igf2* imprinting is compromised later in gestation by $p57^{Kip2}$-induced defects.

BWS patients often display specific organomegaly, most often affecting the tongue and adrenal glands, and less frequently the liver, kidney and heart. To distinguish specific organ

overgrowth from generalized overgrowth in *p57H19* mutant mice, we calculated organ weights at 18.5 d.p.c. as a percentage of total body mass. By this criterion, neither *H19_13* nor $p57^{Kip2}$ mutant mice showed specific organ overgrowth (data not shown). In *p57H19* offspring the only organ that displayed significant overgrowth was the tongue which was 122% of wildtype ($p<0.001$) (Table 2). If we tabulate those animals whose normalized growth was one standard deviation above the mean of their wild-type littermates, overgrowth of the kidney, heart, as well as overall somatic overgrowth, are observed more frequently in *p57H19* mutants than in wild-type littermates (Table 3).

D. Placental defects

The most dramatic overgrowth phenotype observed in *p57H19* embryos was placentomegaly, with 18.5 d.p.c. placentas weighing on average 190% of those of wild-type littermates. On histological analysis, mutant placentas were highly disorganized in the labyrinthine layer, where both $p57^{Kip2}$ and *Igf2* are expressed. Mutant placentas also displayed fibrin cysts, apoptotic cells and large accumulations of red blood cells. A similar disorganization was seen at 16.5. and 17.5 d.p.c., suggesting that this effect occurs before the degeneration of the organ that normally occurs in late gestation. It is unclear whether red blood cells accumulate because of the tubule networks through which maternal and fetal blood flow are not established, are breaking down through cell death, or are blocked by surrounding cellular overgrowth. $p57^{Kip2}$ single mutant animals showed related morphological placental defects, including reduced vascularization of the labyrinthine zone caused by an overproliferation of trophoblast cells. In addition, the occurrence of hyaline membranes, a response to endothelium damage, was observed and was thought to result in a blockage of the blood supply [(121). The *p57H19* phenotype that we observed, however, was far more severe.

In *p57H19* mutants there was a direct correlation between the placental weights and disruptions in placental cellular architecture, suggesting that the observed placentomegaly is caused primarily by the increase in red blood cell volume. Interestingly, there was actually a modest positive correlation ($r=0.37$) between the disorganization of the placenta, and the size of the embryo, as reflected by its weight. Thus the placental dysmorphologies did not compromise fetal growth.

The placental morphology of BWS patients has been examined in a few cases where disease was anticipated prenatally or following stillbirths or neonatal death. In these cases, placentas contained large cysts in stem villi. Some of the terminal villi in individual cases were filled with blood, and the trophoblast layer was hyperplastic in some, but not all, cases (122). Despite the significantly different architectures of the human and mouse placentas, these phenotypes are similar to those we observe in the *p57H19* mice.

E. Kidney Dysplasia

Kidneys in *p57H19* mice were underdeveloped in the medullary region, where $p57^{Kip2}$ is normally expressed. This region normally consists of stromal mesenchymal cells surrounding a duct collection system. In the mutant mice, fewer collection ducts and dilated renal pelves were observed. Kidneys varied from normal to kidneys in which there was no evident medulla or which contained large cystic-looking regions disrupting the medulla. In general, the degree of disorganization in the medulla was significantly more pronounced than had been observed in the $p57^{Kip2}$ single mutant strain, where slightly fewer collection ducts, less mesenchymal tissue and a reduced medulla as well as more stromal cells were reported (120).

There was a strong correlation between the severity of the placental and kidney phenotypes within individual *p57H19* mutant embryos. It is possible that the disorganized placenta, through ineffective nutrient transfer, results in the defects in kidney development. Alternatively, both tissues might independently be sensitized to excess IGF2 in the absence of $p57^{Kip2}$ expression. The variability in the kidney phenotype was observed between animals, not between the two kidneys of a single animal, suggesting that genetic background could play a critical role in its severity.

Renal dysplasia is a common feature of BWS. Specifically, patients with a reduction in the collecting ducts and numerous fluid filled cysts as well as hypertrophy of the mesenchymal tissue have been reported. In humans, renal dysplasia is thought to result from abnormal metanephric

differentiation (123). We did not observe hypertrophy in *p57H19* kidneys, which may reflect the fact that most BWS kidneys analyzed are from adults, not fetuses. In all other respects, *p57H19* dysplastic kidneys appear to mimic most of the aspects of the human disease. In contrast to the kidney dysplasias, we saw no defects in adrenal architecture or size in *p57H19* mice. This was surprising in light of the previous finding of adrenomegaly in $p57^{Kip2}$ mice (120).

F. Abdominal Wall Defects

Closure of the ventral abdominal wall in mice occurs around 16.5 d.p.c. and is preceded by the retraction of the midgut into the abdominal cavity. Failure of the intestine to retract results in an umbilical hernia whereas failure of the abdominal wall to close results in an omphalocele, in which the midgut protrudes from the abdomen. Around 60% of BWS patients need surgical correction of omphalacele at birth, andanother 32% display umbilical hernias (Table 3). One or the other of these phenotypes was seen in 55% of *p57H19* embryos, consistent with the previous report that $p57^{Kip2}$ mutants display omphalocele as well as body wall muscle dysplasia. Although this condition is not observed in *H19*Δ13 mutants, it is seen in *Igf2r*$^{-}$ and *Igf2r*$^{-}$*/ H19*Δ13 double mutants, where IGF2 levels are elevated >2-fold (115,116,117,114).

G. Cleft Palate and Skeletal Abnormalities

BWS patients occasionally display cleft palate, a condition that results from the failure of the palatal shelves to elevate, rotate or fuse. Previous studies had detected cleft palate in *Igf2r/H19Δ13* as well as in $p57^{Kip2}$ mutant mice but not in either *H19Δ13* or *Igf2r*$^{-}$ mutant mice, implying that either significantly elevated IGF2 or reduced p57 could lead to the developmental defect. In *p57H19* mutants, the frequency and severity of cleft palate, which was detected in 26% of offspring, was similar to that seen in $p57^{Kip2}$ single mutants (Table 3), suggesting that the primary cause of cleft palate is loss of $p57^{Kip2}$ function. It has been proposed that the cleft palate in $p57^{Kip2}$ mutants results from the failure of $p57^{Kip2}$ mutant cells to exit the cell cycle, and to undergo apoptosis (119).

Polydactyly has been observed in ~5% of BWS patients (Table 3). This phenotype has also been observed in mice displaying elevated IGF2, with the frequency and severity increasing with the dosage of IGF2. $p57^{Kip2}$ single mutants do not display polydactyly whereas *H19Δ13*$^{-}$ mice do display postaxial polydactyly 68% of the time on a 129/Sv background (114). As expected for an *Igf2*-dependent phenotype, *p57H19* double mutants also show postaxial polydactyly in two genetic backgrounds examined (Table 4).

H. The Role of Igf2 in the p57H19 phenotype

The dramatic increase in the severity of kidney and placental dysplasia in *p57H19* double mutants occurred in animals over-expressing IGF2. Although the somatic overgrowth observed in *H19Δ13* mutant animals was genetically shown to be the consequence of the over-production of IGF2 (113), it is possible that the novel defects observed in *p57H19* mice are due to effects of the *H19* deletion on genes other than *Igf2* or possibly even due to the loss of the *H19* mRNA itself. If the new phenotypes are due to IGF2 overproduction, a reduction in IGF2 levels should ameliorate them. To test this, *p57H19* females were crossed to $Igf2^{+/-}$ males with a null allele of *Igf2* (*p57H19; Igf2*$^{-}$) (91). Previous studies had determined that while the expression of *Igf2* mRNA from the maternal chromosome in *H19Δ13* mice in mesodermal tissues such as skeletal muscle and heart was essentially equivalent to that on the paternal chromosome, its derepression was less pronounced in endodermal tissues such as liver (113). Furthermore, the circulating levels of IGF2 were only modestely elevated (114). Thus overall maternal expression of IGF2 in *p57H19; Igf2*$^{-}$ mutants is lower than that from a wild-type paternal chromosome.

At 18.5 d.p.c. triple mutant fetuses (*p57H19; Igf2*$^{-}$) were indistinguishable in size from wild-type, or *p57H19* fetuses (Table 2). In contrast, $Igf2^{+/-}$ offspring were 58% the size of wild-type, as had been observed previously (91). Thus in the absence of $p57^{Kip2}$, prenatal growth was relatively

insensitive to the ~2-fold differences in the levels of IGF2 between *p57H19* and *p57H19;Igf2*$^{-}$ mice. The complete absence of IGF2 in *p57*$^{Kip2\ -/+}$; *Igf2*$^{+/-}$ double mutants, on the other hand, results in embryos that are the same size as their *Igf2*$^{+/-}$ litter-mates (data not shown).

In contrast to the insensitivity of somatic growth to the reduction in *Igf2*, the placental overgrowth and dysplasia in *p57H19* embryos were almost completely suppressed in triple mutants (Table 2). That is, the mean weight of placentas did not differ from that of wild-type littermate placentas, but did differ substantially from that of the *p57H19* placentas, which were 53% over-sized in this cross. On histological examination, triple mutant placentas were morphologically normal, lacking even the moderate level of disorganization seen in *p57*^{Kip2} single mutants. Thus the placental dysplasia, which is *p57*^{Kip2}-dependent, can be suppressed by reducing the levels of IGF2.

The same situation appears to hold for the kidney dysplasia. None of the triple mutants had cystic kidneys, and the only discernible differences observed in a few fetuses were a slightly less developed medulla and a slight increase in mesenchymal tissue between the renal tubules. However, inmost animals, the kidneys were less affected than in p57KIP2 animals. Finally, the macroglossia observed in the p57H19 mutant was completely absent in the triple mutant, indicating that overgrowth of the tongue is also Igf2 dependent (Table 2). This is consistent with the finding of macroglossia in Ifg2 overexpressing transgenic mice (118).

The frequency of abdominal wall defects in *p57H19* mice was unaffected by reducing the levels of *Igf2* expression in *p57H19; Igf2*$^{-}$ mice (Table 4). This result is somewhat surprising, as omphalocele was induced in the presence of highly elevated levels of IGF2 in *Igf2r*$^{-}$ and *Igf2r*$^{-}$*/H19* mutant mice. Thus, in *p57H19* mice, this defect appears to be entirely attributable to *p57*^{Kip2} loss-of-function. Likewise the frequency of cleft palate is not affected by a loss of *Igf2* in the triple mutant, even though cleft palate was observed in *Igf2r*$^{-}$*/H19* double mutants. Lastly the postaxial polydactyly that is observed in both p*57H19* and *H19* mutants was completely rescued by the reduction in *Igf2* expression.

I. p21$^{-/-}$p57$^{-m/+}$ double mutants show altered lung development. Histopathological examination of p21$^{-/-}$p57$^{-m/+}$ mice revealed all of the phenotypes caused by p57$^{-p/+}$ loss alone and several novel phenotypes in tissues that are apparently unaffected in either of the single mutant animals. Unlike p21$^{-/-}$ or p57$^{+/-m}$ animals, the lungs of p21$^{-/-}$p57$^{-m/+}$ animals were clearly defective, failing to fully differentiate distal air sacs, the ultimate functioning unit for gas exchange in lung tissue. The mammalian lung is composed of two types of tissues, an epithelium that lines all the airways from the trachea to alveoli and a mesenchymal stroma that supports the epithelium. Lung development is divided into several periods. In the pseudoglandular period early during embryogenesis, the lung resembles an exocrine gland and consists of a complex of branching bronchial tubes that include the primary, secondary, segmental and terminal bronchi, and the bronchioles. This is followed by the canalicular period when respiratory bronchioles are formed. Each respiratory bronchiole is terminated in two or three thin-walled dilations termed terminal sacs or primitive alveoli. At E16.5, lungs from WT embryos display substantial formation of primitive alveoli manifested as "open spaces" on H&E stained sections. In contrast, lungs from p21$^{-/-}$p57$^{+/-m}$ animals are virtually devoid of "open spaces". Under high magnification, it is evident that primitive alveoli do not develop in the double mutants. This defect persists until birth. Furthermore, there is a decrease in the size of the lumenal space of the bronchi and bronchioles in the double mutants. p21$^{+/-}$ p57$^{+/-m}$ lungs exhibit an intermediate phenotype between the WT and the double mutant with some primitive alveoli but fewer than in the WT, indicating a single p21 gene is insufficient in the absence of p57.

To explore the cause of the lung defect, we examined the expression of both p57 and p21 in the developing lung. p57 is highly expressed in bronchiole epithelium mirroring that of CC10, a marker for that tissue. p57 is expressed at lower levels in an undefined subset of lung mesenchymal cells and the epithelium lining of the terminal primitive alveoli.

In contrast, p21 is expressed throughout the lung. Despite high levels of expression of p57 in the bronchiole epithelium, no significant abnormalities were detected in this tissue and tissue specific differentiation markers such as CC10 and SP-A, B and C are expressed normally in the double mutants. Although the absence of air sac lumenal space gives the appearance of increased cellularity in the mutants, this is not the case. This is due to the fact that the lungs of the double mutant mice are smaller than the wild-type lungs, thus the total number of cells are approximately the same. Furthermore, the overall proliferation rates in the double mutant lung were not elevated as judged by BrdU pulse labeling nor was there an increase in apoptosis. Thus, the defects in primitive alveoli formation in the absence of p21 and p57 is likely to result from subtle changes in the differentiation of either the epithelia or the mesenchymal stroma for which additional studies are required to delineate more precisely.

J. Skeleton defects in $p21^{-/-}$ $p57^{-m/+}$ double mutants. The only phenotype of $p57^{+/-m}$ mice that is enhanced by loss of p21 is the skeletal phenotype. Deletion of p57 alone causes delay in ossification and sternal fusion defects, but no overall abnormality in the shape of the skeleton. However, $p21^{-/-}$ $p57^{-m/+}$ double mutant embryos display a posture clearly distinct from those of WT and $p57^{-m/+}$ mutants. Skeleton staining revealed that double mutants lack the spinal curvature seen in WT and $p57^{-m/+}$ single mutants, which might stem from defects in musculature (see below). Rib cage shape in double mutant embryos is also abnormal. Bifurcation of ribs was observed in double mutants, usually of the 9th rib although occasionally the 7th rib is also affected. The femurs of double mutant lack a cartilage outgrowth seen in either p21 or p57 single mutants or WT littermates. The double mutants exhibited sternum fusion defects similar to those seen in p57 single mutants, but the sternum of double mutants is shorter than that of p57 single mutants. The ribs of double mutants join the sternum at an angle of 90°, while the ribs of WT or p57 single mutants join at an angle much less than 90°. Both p21 and p57 have been found highly expressed in developing ribs. However, it is difficult to distinguish autonomous vs. nonautonomous roles of these two inhibitors in ribs, especially considering the fact that similar defects in the attachment of ribs to sternum are observed in mice lacking myogenin.

K. $p21^{-/-}p57^{-m/+}$ double mutants exhibit a profound defect in skeletal muscle. Both p21 and p57 proteins are highly expressed in skeletal muscle but neither single mutant animal showed significant muscle cell differentiation defects. However, $p21^{-/-}p57^{-m/+}$ double mutants exhibit profound defects in skeletal muscle development. We have found no significant difference in skeletal muscle development between $p21^{+/+}$ $p57^{+/-m}$ and $p21^{+/-}$ $p57^{+/-m}$ mice, indicating that a single copy of the p21 gene can fully support skeletal muscle development. As shown by hemotoxylin and eosin staining of transverse sections of E18.5 embryos, the intercostal muscle is greatly reduced in double mutants, and the head muscle is diminished. In the hind limb, numerous long myotubes are observed in $p21^{-/-}p57^{+/+}$ embryos, but many fewer and shorter myotubes are present in double mutants. Defects in the tongue muscle were somewhat less severe and double mutant animals exhibit slightly disorganized and less dense muscle mass when compared to $p21^{-/-}$ $p57^{+/+}$ animals.

The diaphragm and body wall muscles of double mutants are also severely diminished as demonstrated by immunofluorescence staining using a monoclonal antibody against myosin heavy chain (MHC). The root of the diaphragm in double mutants is much thinner and poorly stained by the antibody relative to the WT control. MHC staining was diminished in the diaphragm of double mutants when compared to the WT. In the body wall, WT embryos display three layers of skeletal muscle each of which is diminished in the double mutants.

It is possible that the skeletal muscle defects observed in the double mutants arise from defects in primary myogenesis by which myoblasts are specified and migrate out of somites to various places in the embryo to form skeletal muscles later during secondary myogenesis. At E13.5, a time when primary myogenesis is well underway, however, we observed similarly patterned skeletal muscle groups in the double mutant when compared to a WT embryo. In addition, no difference in the morphology of somites are detected between double mutants and WT animals at E9.5. Therefore, we conclude that the skeletal muscle defects in the double mutants are a result of problems in secondary myogenesis, similar to the defects observed in mice lacking myogenin.

Aim 4. Analysis of the QT domain.
We screened for proteins that bind to C-terminus of p57 by the two hybrid system. In summary, we screened 2 different 2-hybrid cDNA libraries for proteins that could specifically bind p57. One library was prepared from human breast using cDNA from a reduction mammoplasty, the second was a lymphocyte cDNA library we have used previously. We screened over a million transformants for each library, purified out the plasmids that gave a positive signal and performed a secondary screen for those that interacted only with p57. All of the clones isolated from both libraries also activated non-specific baits meaning they were not specific for p57. Therefore, this project was not pursued further.

Aim 5. Transcriptional control of $p57^{KIP2}$.
We completed this aim and discussed it in last years report. We did not publish a paper on our findings yet. Basically we have identified a 5 kB region of the p57 promoter that when fused to lacZ and used to generate transgeneic mice expresses the lacZ gene in many of the tissues that p57 is normally expressed in. Therefore, we think we have isolated key regulatory elements of the promoter and are in the process of delineating specific regulatory elements in this 5 Kb fragement.

Aim 6. Identification of new CKIs and other potential regulators of Cdks from normal breast. This aim proposes to look for additional Cdk binding proteins in the breast using the two hybrid system and breast cDNA libraries. Our screens did not turn up any new proteins that were not already identified in previously published screens usinglibraries from other tissue sources.

In summary, we screened 2 different 2-hybrid cDNA libraries for proteins that could specifically bind Cdk2. One library was prepared from human breast using cDNA from a reduction mammoplasty, the second was a lymphocyte cDNA library we have used previously. We screened over a million transformants for each library, purified out the plasmids that gave a positive signal and performed a secondary screen for those that interacted only with Cdk2. Several clones were identified that specifically recognized Cdk2 but all were previously identified CKIs or other Cdk binding proteins such as cyclins. Therefore we did not pursue this aim further.

Key Research Accomplishments

The cell cycle is regulated by the action of a family of cyclin dependent kinases (Cdks) which catalyze particular cell cycle transitions. Cdks are positively regulated through interaction with cyclins and are negatively regulated through phosphorylation and through association with inhibitory proteins of the CIP/KIP and INK4 families. Our research has focused on the role of p57KIP2 in development and cancer. We have found that p57KIP2 is expressed in a highly cell type specific manner during embryonic development and in adult tussues, being most highly expressed in terminally differentiated cells. Through analysis of p57KIP2 deficient mice, however, we have found that p57KIP2 is required for normal development of several tissues including the kidney, lens, muscle, and bone. We also discovered the p57 is involved in the human overgrowth and cancer predisposition disease Bechwith-Weidemann Syndrome, BWS. Current studies are aimed at understanding in greater detail the function of p57KIP2 and its possible role in human cancers.

Reportable Outcomes

Caspary, T., Cleary, M.A., Perlman, E.J., Zhang, P., Elledge, S.J., and S.M. Tilghman. (2000) Oppositely imprinted genes p57(Kip2) and igf2 interact in a mouse model for Beckwith-Wiedemann syndrome. *Genes Dev.* 13:3115-24.

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Conclusion:

The last four years was a very productive period for our lab and the cell cycle field in general. Our work funded under this grant allowed us to establish the role of $p57^{KIP2}$ in mouse development and the human cancer and overgrowth syndrome BWS. $p57^{KIP2}$ clearly acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia, and certain chondrocytes. The partial dependency on $p57^{KIP2}$ for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and Cdks. While undergoing the process of reducing Cdk activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before Cdk activity is sufficiently reduced to block cell cycle entry. In addition, other CKIs may provide Cdk inhibitory functions in the absence of $p57^{KIP2}$, as we have shown here in the lens development of p27/p57 double mutant mice.

CKIs are the ultimate effectors of signal transduction pathway intended to bring about cell cycle arrest and the patterns of expression during embryonic development suggest that particular *CKIs* play important roles in terminal differentiation in a tissue specific manner. However, the fact that mice lacking single *CKIs* display surprisingly few developmental phenotypes has brought into question the essential nature of *CKIs* for cell cycle arrest and differentiation. Our studies **funded by this grant demonstrate** that two *CKIs*, *p57* and *p21* cooperate to control proliferation and differentiation in multiple tissues and reiterates the critical importance of *CKIs* to cell cycle control during development. The use of multiple *CKIs*, each controlled through distinct signaling pathways, provides a flexible mechanism to control proliferation in a cell type specific manner. It is likely that the combinatorial use of *CKIs* will emerge as one of the principal means through which cell cycle arrest and differentiation are integrated during development.

The more recent studies on the roles of IGF2 and p57 was prompted by the clinical findings in BWS that mutations in $p57^{KIP2}$ and over-expression of *IGF2* have each been proposed as causes of the disease. Yet the phenotypes of the mouse models for loss of function of p57KIP2 and loss of imprinting of Igf2 are readily distinguished (113, 119, 120, 124) McLaughlin et al (124) have shown that mice containing a UPD of a large portion of the distal end of mouse chromosome 7 die at 9.5 d.p.c., presumably as a consequence of the reduced expression of *Mash2,* a gene that is required for placental development and is maternally expressed (125, 126). By creating a mouse model in which only the two candidate genes are affected, we hoped to gain more precise insight into the ways in which mis-regulation of these two apparently unrelated genes could lead to the same disease.

The *p57H19* mice exhibited a dramatic increase in the severity of several BWS phenotypes such as placental overgrowth and dysplasia and kidney defects. Furthermore, macroglossia, one of the hallmarks of BWS, was seen in these mice. A direct interaction between the $p57^{Kip2}$ and *Igf2* pathways is implied by the ability of the *Igf2* mutation to compensate for the placental and kidney dysplasias that arise from mutations in $p57^{Kip2}$. Thus in the absence of $p57^{Kip2}$, the disorganized development of those tissues is enhanced in an *Igf2*-dependent manner. When signaling through *Igf2* is reduced, the loss of $p57^{Kip2}$ is no longer detrimental. Thus our analysis of *p57H19* mice suggests a resolution to the dilemma of how both loss-of-function mutations in $p57^{KIP2}$ and gain-of function mutations in *IGF2* can lead to BWS. That is, in the mouse the two genes act in an antagonistic manner in a subset of the tissues in which they are co-expressed.

A pathway that is affected by both genes is the one that regulates G1 cell cycle progression. Both IGF2 and p57 are involved in regulating the progression of cells through the G1/S phase of the cell cycle, with IGF2 promoting the G1/S transition (127) and p57 inhibiting the G1 cyclin-dependent kinases (CDKs) (89, 90). p57 has not been implicated in regulating embryo size, but is involved in the cell cycle arrest that precedes terminal differentiation of tissues such as skeletal muscle, lens and placenta (120, 121, 128). IGF2, on the other hand, is a direct regulator of fetal growth, and has been shown to promote progression through the G1 phase of the cell cycle, possibly through its ability to increase the level of the G1 cyclin D1 (127). The decision to proceed through the G1 to S phase

transition checkpoint is controlled by the ratio of cyclin/Cdk complexes to CKIs, which determines the overall activity of G1-cyclin/Cdk complexes. It is possible that the exacerbation of the $p57^{Kip2}$ phenotype in the presence of excess IGF2 and its alleviation when the concentration of the growth factor is reduced reflects cell-type specific sensitivity to Cdk activity in placenta and kidney. Excess IGF2 in the absence of $p57^{Kip2}$ could lead to hyperproliferation, as seen in the placenta, to increased apoptosis, which is observed in both $p57^{Kip2}$ and *p57H19* mutant placentas and kidneys, or to a failure to differentiate, as is suggested by the reduction of medullary cells in the kidney. Increased apoptosis has also been reported in the palatal shelves of $p57^{Kip2}$ single mutants, and presumably results from alterations in the orderly progression through cell cycle checkpoints (119).

Several $p57^{Kip2}$-dependent phenotypes such as cleft palate and omphalocele are neither enhanced by over-expression of *Igf2*, nor rescued by its absence. It may be that the insensitivity of cleft palate and omphalocele phenotypes to changes in IGF2 levels reflects the fact that these tissues regulate the CDK to CKI levels using growth factors other than IGF2. On the other hand both cleft palate and omphalocele are observed in the most severe IGF2 gain-of-function mouse model, the *Igf2r*/H19 double mutant, where IGF2 levels were 7-11 fold higher than normal (114). These animals also displayed the lens abnormalities and skeletal defects that were seen in the $p57^{Kip2}$ mutants but are not associated with BWS. Thus it may be that in the presence of $p57^{Kip2}$, very high levels of IGF2 are required to alter the CDK to CKI balance.

One of the surprising findings in this study was that the somatic overgrowth that has been shown to be *Igf2*-dependent in *H19Δ13* mice was less pronounced in *p57H19* mice. We considered the possibility that the lack of somatic overgrowth was due to the failure of the dysplastic placenta to provide nutrients to the embryo in the later stages of gestation. However the subset of *p57H19* mice in which the placenta was relatively normal were not oversized at birth (data not shown). Furthermore there was a modest positive correlation between the degree of placental hyperplasia and the size of the embryos in general (r=0.37). The fact that $p57^{Kip2}$ mutants are reduced in size in the presence of a null mutation in *Igf2* argues that the $p57^{Kip2}$ mutation does not completely desensitize the embryo to changes in IGF2 concentration. Rather it may be that the~2-fold changes in IGF2 expression between $p57^{Kip2}$, *p57H19* and *p57H19; Igf2* mice do not shift the ratios of CDKs to CKIs sufficiently to effect a change in overall growth rate. Of the Cip/Kip family of CKIs, only $p27^{Kip1}$ has been directly implicated in overall fetal growth (129, 130, 131, 132).

Our results suggest a model in which p57 and IGF2 act antagonistically in the control of cell proliferation and development in several tissues affected in BWS patients including the tongue, kidney, and placenta. The affected tissues in the mouse are those in which some rate-limiting step is controlled by the two growth regulators, while other tissues, like the liver, presumably utilize other positive or negative growth signals. Although the nature of these other pathways are unknown, IGFI has been shown to function redundantly with IGF2 to promote cell growth (133). Likewise several instances of redundancy between Cdk inhibitors have been observed. For example, p21 and p57 are redundant for control of muscle and lung development (128); p27 and p57 are redundant for control of lens development (121); and p18 and p27 work together to control pituitary gland, spleen, and thymus embryonic growth (134). We propose that BWS phenotypes are observed when the overall balance of regulators are shifted in favor of proliferation by either an increase in IGF2, or a decrease in p57. While a shift in the balance of other regulators could potentially cause a BWS-like phenotype, it is likely that the differing tissue specificity of the other regulators would prevent them from producing a phenotype recognized as BWS.

The fact that the *p57H9* double mutant does not completely recapitulate the BWS phenotype probably reflects species-specific differences in the tissues in which the two genes act directly in opposition to one another. Furthermore because the changes in the cell number in a given organ due to a change in the rate of proliferation is an exponential function, the more rounds of cell division a tissue undergoes, the greater will be the effect of a small increase in proliferation rate. Since human organs such as the tongue, kidney and placenta undergo significantly more cell division, a relatively small

change in proliferation rates afforded by p57 loss or increased IGF2 may have a more pronounced effect in humans relative to the mouse.

The variability in the phenotypes in the *p57H19* mice is reminiscent of the highly variable phenotypes of BWS patients (Table 3). The mice in this study were not on completely inbred backgrounds, and at least some of the variability we observed could be attributed to genetic modifiers. Another explanation for the variability in the human syndrome is suggested by the curious clinical finding that 10 sets of female identical twins have been reported who are discordant for BWS (135). Although the high level of discordancy is not understood, it has been suggested that disruptions in epigenetic mechanisms such as X-inactivation and imprinting might explain the occurrence, an explanation that could extend to sporadic cases as well.

In conclusion the analysis of the defects in *p57H19* mice demonstrates that some but not all tissues are highly sensitive to the ratio of p57 and IGF2. Perturbations in the levels of either protein may be sufficient to generate the variable range of phenotypes in BWS.

The studies performed in this grant have suggested that loss of p57 is unlikely to cause breast cancer as based on the phenotypes of patients with BWS who have a mutant copy on the expressed allele of p57. These individuals do not have increased incidence of breast cancer as far as it has been reported. We cannot state that loss of p57 later in development is not contributory, but is is unlikely given the phenotypes of BWS patients. Still it cannot be ruled out. It is our feeling that p57 may contribute ina small way to certain types of breast cancer that deregulate imprinting at 11p15.5 but this would be a minor percentage of tumorsa and there is no way to define the role played by p57 in such cases since many genes at that locus are likely to be affected in these cases.

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Appendix

Tables 1-4

Table 1. Genetic crosses

Cross	# litters	Genotype	#embryos
p57H19$^{+/-}$ X C57BL/6	24		170
		p57H19	99
		+/+	71
C57BL/6 X *p57H19*$^{+/-}$	6		52
		p57H19	12
		+/+	14
p57H19$^{+/-}$ X *Igf2*$^{+/-}$	10		75
		p57H19	14
		p57H19; Igf2$^{+/-}$	26
		Igf2$^{+/-}$	15
		+/+	20
H19Δ13$^{+/-}$ X *C57BL/6*	4		26
		H19Δ13$^{+/-}$	12
		+/+	14
p57$^{Kip2+/-}$ X C57BL/6	4		30
		p57$^{Kip2+/-}$	16
		+/+	14
p57$^{Kip2+/-}$ X *Igf2*$^{+/-}$	2		22
		p57$^{Kip2+/-}$	2
		Igf2$^{+/-}$	7
		p57$^{Kip2+/-}$ *;Igf2*$^{+/-}$	6
		+/+	7

Embryos of the crosses indicated were genotyped between 12-18.5 d.p.c. Female parent indicated first.

Table 2 *Growth of p57H19 mutant embryos*

Tissue	P57H19 (BL/6) n = 20	p57H19 (BL/6-129) n = 8	$Igf2^{+/-}$ (BL/6-129) n = 10	p57H19; $Igf1^{+/-}$ (BL/6-129) n = 19
Embryo	1.04	1.06	0.58*	0.93
Placenta	1.94*	1.53*	0.55*	1.0
Tongue	1.22*	1.11	0.86	1.00
Heart	1.03	1.17	1.03	1.16
Kidney	1.13	N.D.	N.D.	N.D.
Liver	0.83	0.98	0.75	0.89

The wet weights of embryos and internal organs of the genotypes indicated are expressed as a fraction of that of wild type littermates. The p57H19(BL/6) amimals were derived from crosses to C57BL/6 males; all others were derived from a $p57H19^{+/-}$ x $Igf^{+/-}$ cross. (N.D.). Not determined. *$P < 0.05$.

Table 3 *Summary of phenotypes in BWS animal models*

Phenotypes	Percent Observence		
	BWS	p57H19	+/+
Macroglossia	95	60(12/20)	11(1/9)
Adrenal defects cytomegaly	94	0	0
" cysts	69	0	0
Placentomegaly	92	96(27/28)	13(2/15)
Visceromegaly hepatomegaly	85	16(3/19)	11(1/9)
" nephromegaly	69	40(8/20)	13(1/8)
" cardiomegaly	N.D.	15(3/20)	13(1/8)
Somatic overgrowth	60	39.2(11/28)	13(2/15)
Renal dysplasia	59	61.5(8/13)	0
Abdominal wall defects omphalocele	60	4(1/23)	0
" " umbilical hernia	32	48(11/23)	0
Cleft palate	7.1	26(6/23)	0
Subcutaneous cleft palate	20	N.D.	N.D.
Cardiac defects	20	39(5/13)	0
Polydactyly	5.5	30(7/23)	0

The frequency of the phenotypes is expressed as a percentage of all individuals examined. The BSW data are adapted from Eggenschwiler et al. (1997). The p57H19 and +/+ data are taken from 18 d.p.c. p57H19 x C57BL/6) litters. (N.D.) Not determined.

Table 4. *Effect of reducing IGF2 in p57H19 mice*

	Genotype	
Phenotype	p57H19	p57H19; Igf2
Postaxial polydactyl	50(4/8)	0(0/15)
Cleft palate	12.5(1/8)	20(3/15)
Omphalocele	37.5 (3/8)	20(3/15)
Umbilical hernia	25(2/8)	27(4/15)

The frequency of the phenotypes in 18 d.p.c. fetuses are expressed as a percentage of all individuals examined.

Altered cell differentiation and proliferation in mice lacking $p57^{KIP2}$ indicates a role in Beckwith–Wiedemann syndrome

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Mice lacking the imprinted Cdk inhibitor $p57^{KIP2}$ have altered cell proliferation and differentiation, leading to abdominal muscle defects; cleft palate; endochondral bone ossification defects with incomplete differentiation of hypertrophic chondrocytes; renal medullary dysplasia; adrenal cortical hyperplasia and cytomegaly; and lens cell hyperproliferation and apoptosis. Many of these phenotypes are also seen in patients with Beckwith–Wiedemann syndrome, a pleiotropic hereditary disorder characterized by overgrowth and predisposition to cancer, suggesting that loss of $p57^{KIP2}$ expression may play a role in the condition.

Cell proliferation in the embryo is controlled by an intricate network of extracellular and intracellular signalling pathways that process growth regulatory signals and integrate them into the basic cell-cycle regulatory machinery through control of cyclin-dependent kinases (CDKs). CDKs are positively regulated by cyclins and negatively regulated by CDK inhibitory proteins, CKIs[1,2]. Cyclins D and E function in the G1 phase of the cell cycle and phosphorylate and inactivate the tumour-suppressor retinoblastoma (Rb) and the related proteins p107 and p130, which are negative regulators of the E2F transcription factors that facilitate cell-cycle entry[3]. There are two classes of CKIs in mammals, the $p21^{CIP1}$ and $p16^{INK4}$ families. Members of the family $p16^{INK4}$ bind and inhibit only Cdk4 and Cdk6 kinases. Mice lacking the tumour-suppressor $p16^{INK4}$ show no gross developmental abnormalities but have high rates of spontaneous tumorigenesis[4]. In contrast, $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ CKIs inhibit all G1/S phase CDKs[1,2]. $p21^{CIP1}$ is transcriptionally regulated by the p53 tumour-suppressor in response to DNA damage, and is required for the G1 DNA-damage checkpoint[1,2]. Although $p21^{CIP1}$ expression during development correlates with terminally differentiating tissues[5], mice lacking $p21^{CIP1}$ develop normally[6]. $p27^{KIP1}$-deficient mice are grossly normal developmentally but display several phenotypes that seem to be linked to cell proliferation[7–9]. They are larger than wild-type mice, have intermediate-lobe pituitary hyperplasia or adenomas, and females are infertile.

The most structurally complex member of the $p21^{CIP1}$ family of CKIs is $p57^{KIP2}$ (refs 10, 11), which resides at 11p15.5 (ref. 10), a site of frequent loss of heterozygosity in several human cancers including those of the breast, bladder, lung, ovary, kidney and testicle. Several types of childhood tumours display a specific loss of maternal 11p15 alleles, suggesting the involvement of genomic imprinting[12]. Rearrangements at 11p15 are seen in Beckwith–Wiedemann syndrome (BWS), which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, enlarged adrenal glands, ear creases, visceromegaly (enlarged organs), omphalocele (umbilical hernia), kidney abnormalities, advanced ageing and thickening of long bones, and a 1,000-fold increase in the risk of childhood tumours[13], including Wilms' tumour, adrenocortical carcinoma, and hepatoblastoma. BWS occurs with an incidence of 1 in 13,700 births, and 15% of these are familial with maternal carriers, suggesting a role for genomic imprinting[14]. BWS has highly variable penetrance in which patients usually display only a subset of all phenotypes. BWS has a complex pattern of inheritance including uniparental disomy (paternal), paternal trisomy of chromosome 11p15.5, paternal duplication of the 11p15.5 region, translocations involving maternal 11p15.5 and karyotypically normal transmission.

The protein $p57^{KIP2}$ is encoded by a maternally expressed, imprinted gene in both humans[15] and mice[16], and one cluster of BWS translocations[17] is within 80 kilobases of the $p57^{KIP2}$ gene. A recent study reported that two of nine patients with BWS examined[18] were found to be heterozygous for mutations in the $p57^{KIP2}$ gene. However, there was no analysis of how frequently these alterations were observed in an asymptomatic population. Furthermore, only one patient had an apparent null allele that was shown to be maternally inherited. Because these mutations were found at such low frequency, it is possible that other genes might also be altered in these two patients. To assess directly the role of $p57^{KIP2}$ in development, cancer and BWS, we have generated a mouse lacking the $p57^{KIP2}$ gene. These mice have a variety of developmental defects consistent with a causative role for $p57^{KIP2}$ in BWS, and indicate a role for $p57^{KIP2}$ in control of cell proliferation and differentiation.

Targeted disruption of mouse $p57^{KIP2}$ gene

A targeting construct that removed exons 1 and 2 (Fig. 1a) (87% of the $p57^{KIP2}$ coding region) was introduced into AB2.1 embryonic stem cells. G418/gancyclovir-resistant cells were screened for homologous recombination by Southern blot analysis (Fig. 1b). Homologous recombinant cells were injected into blastocysts from C57BL/6 mice, and male chimaeras were mated to C57BL/6 females. Germline transmission was confirmed by Southern blotting (Fig. 1b). F_1 heterozygous animals were back-crossed to C57BL/6 mice to maintain the disrupted allele. To avoid ambiguity, we indicate the parental origin of alleles in heterozygotes by a superscript m for maternal or p for paternal.

The disrupted allele is a null as demonstrated by the absence of $p57^{KIP2}$ mRNA (Fig. 1e) and protein (Fig. 1c,e) in $p57^{-/-}$ or $p57^{+/-m}$ animals. $p57^{KIP2}$ is detected in the tectum of brain, kidney, adrenal gland, muscle, lung and cartilage in wild-type embryos. Levels of $p21^{CIP1}$ and $p27^{KIP1}$ were unchanged in tissues from $p57^{KIP2}$ mutants, except in muscle where a slight increase in $p27^{KIP1}$ was detected (Fig. 1c). The $p57^{+/-P}$ animals had wild-type expression of $p57^{KIP2}$.

Generation of $p57^{KIP2}$ mutant mice

Of 32 offspring from $p57^{+/-P}$ female to $p57^{+/+}$ male matings, no $p57^{+/-}$ animals were present when genotyped at two weeks of age (Table 1). Of 82 offspring from $p57^{+/-P}$ intercrosses, 55% were $p57^{+/+}$ and 45% were $p57^{+/-}$ (Table 1); no $p57^{-/-}$ animals survived to two weeks of age. Mendelian inheritance predicts a 1:2 ratio for $p57^{+/+}$ to $p57^{+/-}$ animals, indicating that half of the $p57^{+/-}$ animals died before genotyping. We observed dead or dying new-born mice in several litters, and these were found to be mutant. We concluded that $p57^{KIP2}$ is required for postnatal survival in a hybrid C57BL/6-129Sv background. Although lethal in this background, we recently discovered that crossing to the outbred CD1 strain allows some $p57^{+/-m}$ animals to survive well beyond day one.

When evaluated between embryonic day (E)18.5 and E20, genotypes were detected at expected Mendelian frequencies (Table 1). However, 10% of mutant embryos were dead, staged from E13 to E16, consistent with the fact that maternal inheritance of the $p57^{KIP2}$

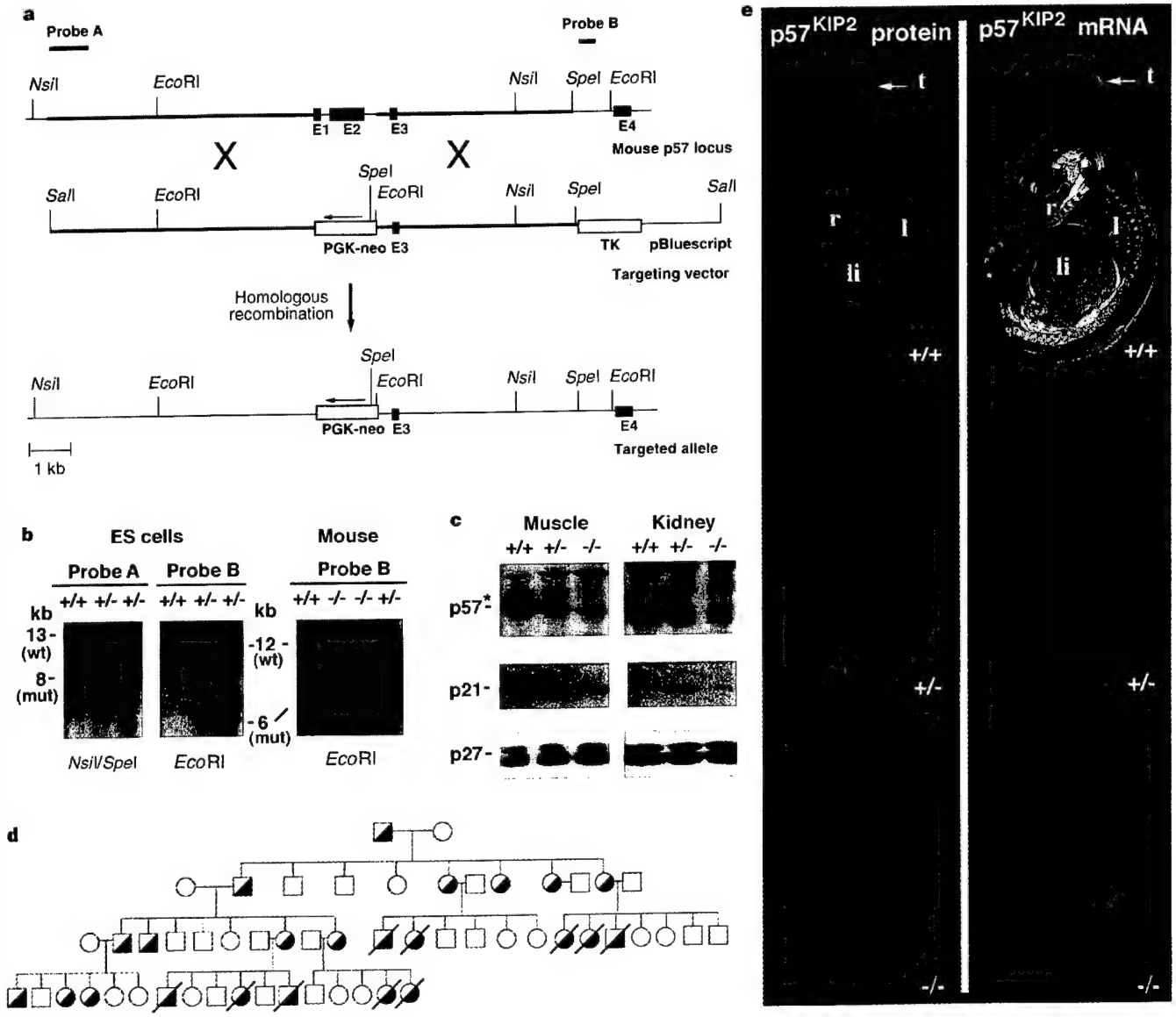

Figure 1 Targeted disruption of $p57^{KIP2}$. **a**, $p57^{KIP2}$ disruption strategy. Probes (A and B) for Southern analysis are indicated. **b**, Southern blot analysis of DNA from wild-type and mutant ES clones and embryos. **c**, Western blot analysis of $p57^{KIP2}$, $p27^{KIP1}$ and $p21^{CIP1}$ proteins in muscle and kidney. The asterisk indicates a form of $p57^{KIP2}$ resulting from phosphorylation or alternative splicing. **d**, A pedigree analysis: squares, males; circles, females. Heterozygotes are represented by half-filled symbols. Animals displaying a mutant phenotype are indicated by diagonal lines through symbols. **e**, *In situ* hybridization and immunofluorescent analysis of sagittal sections derived from E15.5 $p57^{+/+}$, $p57^{+/-m}$ and $p57^{-/-}$ embryos. A combined image of $p57^{KIP2}$ protein (green) and nuclei stained with Hoechst dye (blue) is shown; l, lung; li, liver; r, rib; t, tectum.

null allele is lethal (Fig. 1d). The $p57^{-/-}$ animals were phenotypically indistinguishable from affected $p57^{+/-m}$ heterozygotes.

Mice lacking $p57^{KIP2}$ have omphalocele

Mutant embryos showed umbilical abnormalities as early as E16.5. A herniated abdomen was noticeable in all mutants (Fig. 2A, d,e) together with malrotation of the intestines resulting in placement of the jejunum and ileum in front of the liver. Occasionally, the small intestines were found outside the abdominal cavity (omphalocele), a range of phenotypes characteristic of BWS in humans (Fig. 2A, b). Most dead or dying neonates had a slit in their abdomen where the umbilicus is normally positioned, and portions of their visceral organs were missing, presumably devoured by their mother in the process of removing the placenta and yolk sac (Fig. 2A, c).

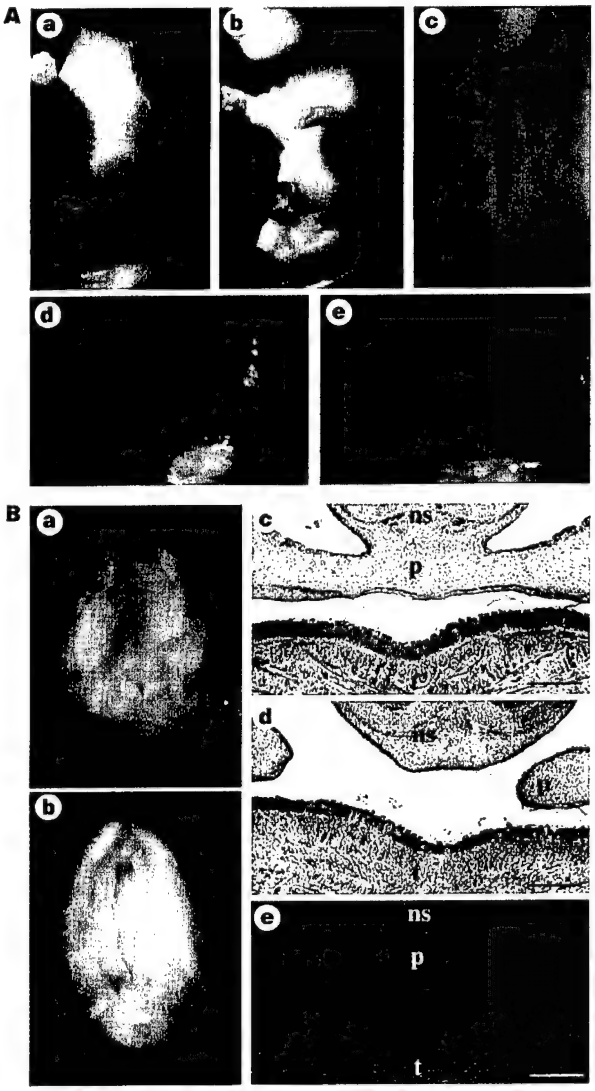

Figure 2 Omphalocele, umbilical hernia and cleft palate in $p57^{KIP2}$ mutant mice. **A**, Intestines are found outside the abdominal cavity in some $p57^{KIP2}$ mutant newborns (**b**) but not wild-type mice (**a**). Umbilical sacs were removed in **b** to expose intestines. Typically, dead neonates (mutants) had a slit on their abdomen with missing viscera (**c**). Umbilical hernia is observed in mutants (**e**, arrow) but not in wild-type littermates (**d**). **a**, **b**, E20; **c**, P0; **d**, **e**, E18. **B**, Jaws were removed from P0 wild-type (**a**) and mutant (**b**) mice to allow palate viewing. Haemotoxylin and eosin-stained coronal sections show a fused palate in E20 wild-type (**c**) mice and cleft palate in mutant (**d**). $p57^{KIP2}$ expression (green) in the palate mesenchyme and tongue muscle of an E20 embryo (**e**); ns, nasal septum; p, palate; t, tongue. Scale bars, 200 μm.

The small intestines develop outside the body until E15.5 (ref. 19), when then they enter the body cavity. How this occurs is unknown. It is unclear how omphalocele occurs in BWS, but the prevailing assumption is that visceral overgrowth limits abdominal space. In $p57^{KIP2}$ mutant embryos, visceral overgrowth was not evident. $p57^{KIP2}$ is highly expressed in intestinal mesenteries that form connections between the intestine folds and the abdomen and may participate in intestine re-entry. However, no obvious histological malformation was detected in mutant mesenteries.

Body wall muscle dysplasia in $p57^{KIP2}$ mutants

E18 mutants are 10% shorter than weight-matched $p57^{+/+}$ embryos (Fig. 3A, a). However, skeletons are nearly identical lengths (Fig. 3A, b), indicating a different aetiology for the body-length anomaly. Because proper musculature is required for skeletal stature, we investigated muscle organization. Histological examination of mutant embryos revealed defects in the position of body wall muscles (Fig. 3B, b,d). At the umbilicus level, the muscle did not reach as far towards the midline of the abdomen in mutants as in wild-type mice, leaving large areas of the abdominal wall uncovered by muscle. To distinguish whether this was a cause or a consequence of herniation, we examined E14.5 embryos in which a physiological omphalocele is normally present. Muscle in the mutants also failed to reach the midline (Fig. 3B, a,c), strongly suggesting that the abdominal wall defect is responsible for the subsequent umbilical herniation.

Overall muscle differentiation was not affected in p57-mutant mice, as indicated by the normal muscle-fibre organization and peripheral location of nuclei. Surprisingly only 50% of muscle nuclei express $p57^{KIP2}$ (Fig. 3B, e,f). This is consistent with the hypothesis that there are two muscle lineages, MyoD and Myf5. Perhaps $p57^{KIP2}$, which is also expressed in E11.5 somites (data not shown), is expressed in only one lineage, and is required for muscle-cell migration.

Neonatal lethality in $p57^{KIP2}$ mutant mice

Cleft palate (secondary palate) and difficulty in breathing were noticeable in all mutant neonates (Fig. 2B, compare a and b). Milk was found in their lungs, and air in their stomach and intestines, causing inflation and stretching. No histological abnormalities were found in mutant diaphragm, lung, bronchi or trachea. The severity of cleft palate was variable but could compromise breathing by allowing an accumulation of liquid in the nasopharynx that is subsequently brought into the lungs by inhalation.

Closure of the secondary palate is a complex process[20]. Vertically growing palate shelves elevate by E13 and grow together, fusing by E16 to form the secondary palate[19]. Analysis of E20 mutant embryos demonstrated that the palate failed to fuse but maintained normal organization (Fig. 2B, c,d). $p57^{KIP2}$ is expressed in mesenchymal cells of the palate and muscle cells of the tongue, but not nasal or oral epithelia (Fig. 2B, e). Failure of palate closure could result from defects in mesenchymal cell migration, response to induction signals, cell proliferation, or increased apoptosis caused by inappropriate proliferation. The tongue of mutant mice is not enlarged, and is therefore not interfering in palate closure.

Renal medullary dysplasia in $p57^{KIP2}$ mutants

A major histopathological finding in BWS is non-cystic medullary dysplasia and enlargement of the kidney[21]. BWS medullary pyramids are often poorly formed and exhibit abundant connective tissue stroma with widely separated renal tubules[22] In $p57^{KIP2}$ mutants the size and organization of the kidney was normal (Fig. 4a–h), but the inner medullary pyramid was significantly smaller than normal. Normally at E18.5 and E20 the maturing glomeruli are juxtamedullary and nephrons have developed long loops of Henle reaching deep into the inner medullary region (Fig. 4g). However, mutants have fewer renal tubules (loops of Henle and collecting

Table 1 Genotype frequencies of offspring

* Male $p57^{+/+}$ × female $p57^{+/-p}$			
p57 genotype	+/+	+/−	
Number	32	0	
Observed (%)	100	0	
Expected (%)	50	50	
* Male $p57^{+/-p}$ × female $p57^{+/-p}$			
p57 genotype	+/+	+/−	−/−
Number	45	37	0
Observed (%)	55	45	0
Expected (%)	25	50	25
† Male $p57^{+/+}$ × female $p57^{+/-p}$			
p57 genotype	+/+	+/−	
Number	24	27	
Observed (%)	47	53	
Expected (%)	50	50	
† Male $p57^{+/-p}$ × female $p57^{+/-p}$			
p57 genotype	+/+	+/−	−/−
Number	16	27	14
Observed (%)	28	47	25
Expected (%)	25	50	25

* Genotyped at 2 weeks of age.
† Genotyped between E18.5 and E20.

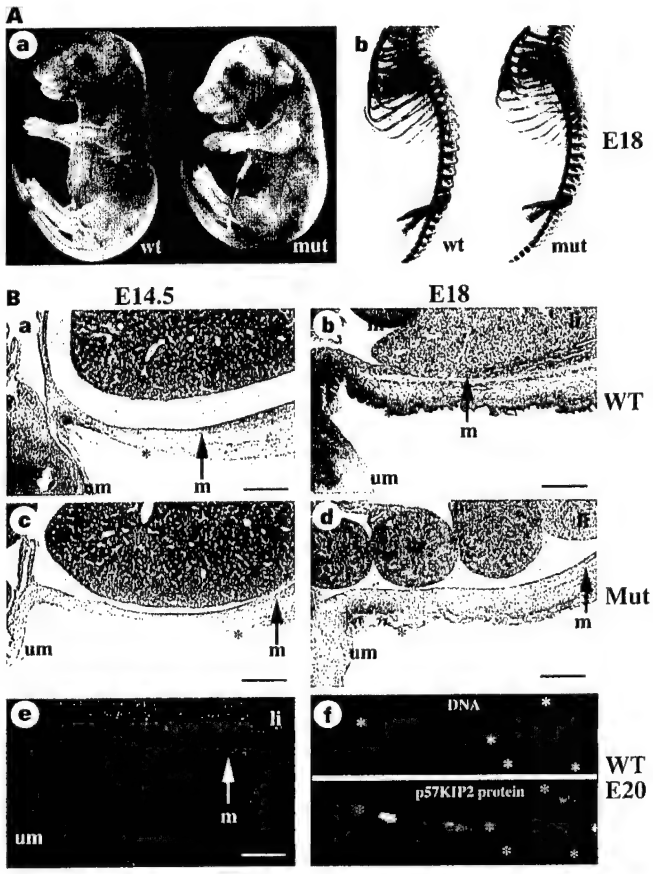

Figure 3 Body-wall dysplasia in $p57^{KIP2}$ mutants. **A**, E18 $p57^{+/+}$(wt) and $p57^{+/-m}$(mut) embryos (**a**) and skeletons (**b**) prepared from the same embryos. Mutants have shorter bodies but normal skeletal length. **B**, Transverse embryo sections were stained with haemotoxylin and eosin. The distance between umbilicus and the tip of rectus abdominis muscle (arrows) is greater in mutants than in wild-type littermates in E14.5 (**a** and **c**) and E18 (**b** and **d**) embryos. Skin development is also delayed in mutants (asterisks). $p57^{KIP2}$ protein was detected in the rectus abdominis muscle, connective tissue, skin, and liver (**e**), and is present in half of the nuclei in muscle (**f**); in, intestine; li, liver; m, muscle; um, umbilical cord. Scale bars, 200 μm.

ducts) and more stromal cells in the inner medulla (Fig. 4d,h), a phenotype very similar to that of BWS patients[21]. No defects in primary nephrogenesis were observed, indicating that ureter bud branching and mesenchymal induction occurred normally in the mutant kidney.

To determine whether medullary dysplasia is due to improper development or to regression of a properly formed kidney, a developmental analysis was performed. At E16 the renal pelvis forms through fusion of initial ureter branches. A normal renal pelvis was observed in the E16.5 mutant kidney (Fig. A, B). At this stage the inner medulla normally begins to form, but in the mutant this was totally absent. At E18.5 the mutant inner medulla has also failed to develop properly (Fig. 4C, D). This indicates that the medullary dysplasia is due to arrested or improper development.

$p57^{KIP2}$ is expressed in podocytes of maturing glomeruli and in interstitial stromal cells between renal tubules (Fig. 4i) but not the tubules themselves. This expression pattern argues against an intrinsic defect in the renal tubule, and underscores the importance of tissue–tissue interactions between epithelia of renal tubules and surrounding mesenchymal cells during the development of the loops of Henle, as has been demonstrated previously for ureteric branching and the mesenchymal to epithelial transition.

Bone development and collagen X expression

Skeletal staining with alcian blue (cartilage) and alizarin red (bone) revealed abnormalities in mutant skeletons. At E14.5 the two sternal bands in mutants were widely separated, whereas those in wild-type animals had already fused (Fig. 5A, a,b). By E19, wild-type sternebrae were fully ossified, whereas ossification had just begun in the mutant (Fig. 5A, c,d). Two separate ossification centres in each sternebra were clearly seen in the mutant, indicating imperfect fusion of sternal bands (Fig. 5A, d). Smaller ossification centres were observed in mutants at all stages of development in forelimbs, vertebra and supraoccipital bone, as shown in Fig. 5A, e–l. Although mutant limbs are shorter, they are thicker than in the wild type (compare Fig. 5A, g and h).

Vertebrate long bones are formed through endochondral ossification, which involves formation of a cartilage framework that is converted to bone by replacement. Within this framework, cells are organized into distinct zones: the epiphyseal centre zone contains resting chondrocytes that act as stem cells for the adjacent proliferative zone, where chrondrocytes proliferate and form columns, and the hypertrophic zone, containing dying chondrocytes that are in the process of ossification. Histological examination of mutant long-bone sections showed a slight disorganization of the columnar alignment of differentiating chondrocytes (Fig. 5B, a,b). Furthermore, the mutant hypertrophic zone is slightly thinner than in the wild type and contains smaller cells, suggesting impairment of chondrocyte differentiation.

$p57^{KIP2}$ is expressed at moderate levels in resting chondrocytes, low levels in the proliferative zone, and very high levels in the hypertrophic zone (Fig. 5B, c). We examined cell-cycle withdrawal in the mutant hypertrophic zone to determine whether cell division in this zone might account for resistance to ossification. A higher level of BrdU incorporation was observed in the resting (2.2-fold) and proliferative (1.6-fold) chondrocytes of E15 mutant animals, but at later times, such as postnatal day (P)0, only a small increase in BrdU labelling was observed in resting (20%) and proliferative (14%) chondrocytes. At E18.5 there is a 10% greater cell density in these two zones, which may explain the bone thickening in mutant animals (Fig. 5A, e–h). However, no BrdU labelling was observed in either the mutant or wild-type hypertrophic zones at any stage.

Collagen X is expressed in hypertrophic chondrocytes and has been implicated in proper bone development[23]. Expression of collagen X was significantly reduced in the mutant hypertrophic zone (Fig. 5B, d,e). Thus $p57^{KIP2}$ is required for expression of collagen X, and perhaps other genes that facilitate the ossification

of chondrocytes. Because no cell-cycle entry was observed in mutant hypertrophic chondrocytes, the failure to express collagen X suggests that $p57^{KIP2}$ may play a direct role in processes of differentiation.

Adrenal cortex hyperplasia and cytomegaly

The adrenal gland is among the most consistently enlarged organs in BWS patients and shows extensive cytomegaly. $p57^{+/-m}$ mutants show a significant enlargement of the adrenal gland (a 25% to 100% increase in volume; Fig. 6a). Although mutant adrenals were normal histologically, there was a threefold increase in the frequency of cytomegaly (Fig. 6c,d). Expression of $p57^{KIP2}$ is restricted to fetal adrenal cortex (Fig. 6b), indicating a role in controlling cell proliferation.

Cycling and apoptosis in lens after loss of $p57^{KIP2}$

The $p57^{KIP2}$ protein is present in high levels in postmitotic lens fibre cells, with low-level, sporadic expression in the anterior epithelial layer (Fig. 7a,b). $p57^{KIP2}$ is induced in the equatorial zone (Fig. 7a, arrows) where epithelial cells are withdrawing from the cell cycle and initiating terminal differentiation[24], suggesting a role for $p57^{KIP2}$ in promoting one or both of these processes. Analysis of $p57^{-/-}$ and $p57^{+/-m}$ lenses revealed grossly normal lens structure at E13.5 with minor vacuolization, but a more pronounced vacuolization in E15.5 and older lenses (Fig. 7e). Late-stage differentiation markers such as γ-crystallin and membrane-intrinsic protein-26 were unaffected (data not shown). However, $p57^{KIP2}$ loss causes inappropriate S-phase entry in lens fibre cells (Fig. 7f,g) and an increase in apoptotic nuclei (Fig. 7i,j), possibly contributing to the vacuolated appearance. Another phenotype of $p57^{-/-}$ lenses was a 10-fold increase in apoptosis in the anterior epithelial compartment (Fig. 7j). Epithelial cells that accumulate in the centralmost position of the anterior epithelial layer are normally eliminated by a p53-independent apoptotic mechanism to maintain the single-cell layer anteriorly[25]. Because these cells express $p57^{KIP2}$, the increase in apoptosis could result from an accelerated rate of proliferation and execution of a normal mechanism designed to eliminate excess cells in the central zone of the anterior lens.

Discussion

$p57^{KIP2}$ acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia and certain chondrocytes. The partial dependency on $p57^{KIP2}$ for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell-cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and CDKs. While undergoing the process of reducing CDK activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before CDK activity is sufficiently reduced to block cell-cycle entry.

Loss of Rb is associated with increases in proliferation, impaired expression of differentiation markers, and inappropriate apoptosis in lens fibre cells[25]. $p57^{-/-}$ lenses show less cell proliferation and apoptosis than $Rb^{-/-}$ lenses, thus $p57^{KIP2}$ is likely to play a partly redundant role upstream of Rb. However, the increase in apoptosis in the anterior epithelial compartment is greater in $p57^{-/-}$ than in $Rb^{-/-}$ lenses. Thus, even in the same cell type, the relationship between $p57^{KIP2}$ and Rb (and possibly other cell-cycle regulators) changes with respect to differentiation state. Furthermore, the ossification defect in $p57^{-/-}$ mice is similar to that observed in $p107^{-/-}p130^{-/-}$ double mutants[26], and it is likely that $p57^{KIP2}$ is also regulating these proteins to control proliferation in chondrocytes.

Several tissues in $p57^{-/-}$ mutants showed developmental defects not obviously linked directly to increased cell proliferation, such as defects in kidney development, differentiation of hypertrophic chondrocytes, muscle organization, and formation of the secondary palate. These defects may indicate roles in differentiation distinct from the biochemical role of $p57^{KIP2}$ as a CKI. In the case of the ossification defects, the hypertrophic chondrocytes exit the cell cycle properly but were disorganized and failed to express differentiation markers such as collagen X. Non-CDK binding regions of the $p57^{KIP2}$ protein, such as the repeat regions or the conserved QT domains, may play cell cycle-independent roles in differentiation.

Using the $p57^{KIP2}$-defective mouse allows us to assess unambiguously the role of $p57^{KIP2}$ in BWS. Its BWS-related phenotypes support a causal role for a mutation found previously[17]. Both patients with $p57^{KIP2}$ mutations displayed omphalocele, macroglossia, gigantism and earlobe grooves. $p57^{KIP2}$-deficient mice also show omphalocele but not gigantism, macroglossia, visceromegaly and hypoglycaemia (data not shown). However, the expression pattern of $p57^{KIP2}$ is consistent with a possible role in both gigantism

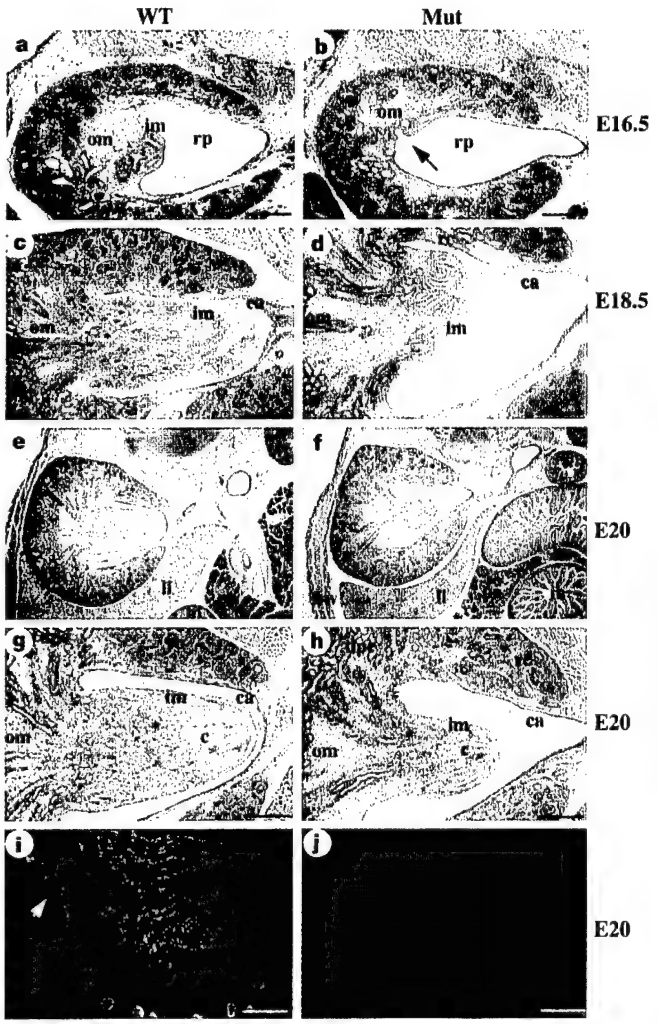

Figure 4 Kidney medullary dysplasia in $p57^{KIP2}$ mutants. **a**, **b**, At E16.5, the inner medulla is absent in the $p57^{-/-}$ mutant kidney (arrow). **c**, **d**, At E18.5, a delayed elongation of the inner medulla is evident in the mutant kidney. **e**, **f**, Low magnification shows relatively normal organization of the mutant kidney at E20. **g**, **h**, A higher magnification of **e** and **f**. The mutant inner medulla is significantly smaller than in the wild type, containing fewer loops of Henle (asterisks) and collecting ducts with more stromal cells in between. (**i**, **j**) $p57^{KIP2}$ protein is expressed in mesenchymal cells between renal tubules of the inner medulla and in podocytes of glomeruli (arrow), but not in mutants (**j**); bw, body wall; c, collecting ducts; dpc, distal and proximal convoluted tuibules; im, inner medulla; in, intestine; li, liver; om, outer medulla; pa, pancreas; ca, renal calyx; rc, renal cortex; rp, renal pelvis. Scale bars, 200 μm.

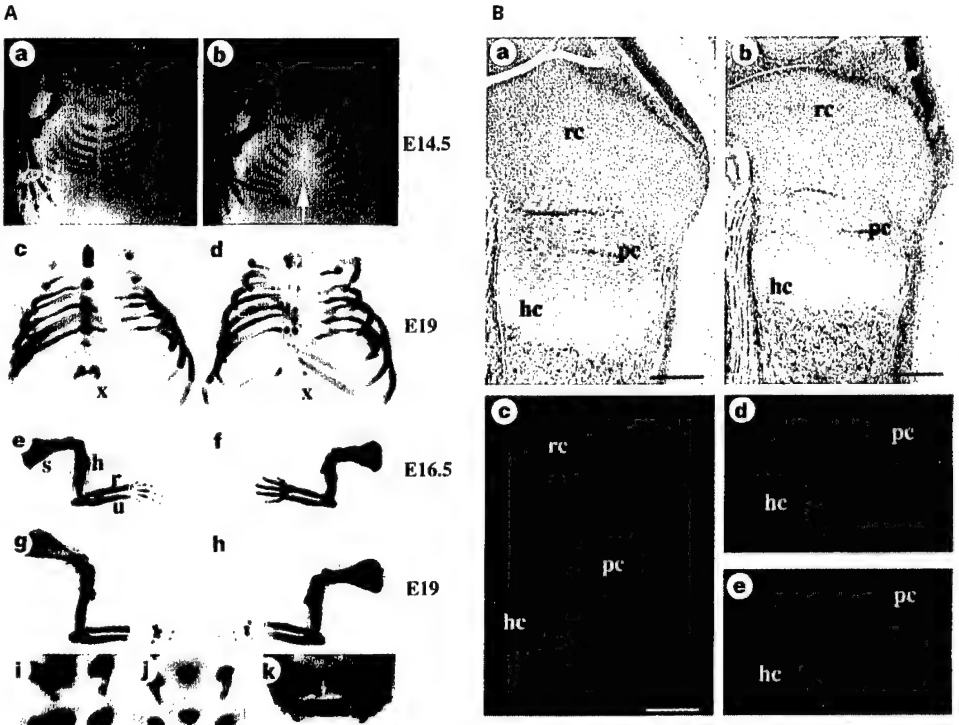

Figure 5 Impaired endochondral ossification in p57^{KIP2} mutants. **A**, Alcian blue (cartilage) and alizarin red (bone) staining of E14.5 skeletons showing fused sternum rudiments in wild-type (**a**) and an unfused sternum in p57$^{+/-m}$ mice (**b**, arrow). Sternebrae are fully ossified in E19 wild-type (**c**) but not mutant (**d**) embryos. Note the separate sternum ossification centres and enlargement of the xiphoid process in **d**. **e**–**h**, Forelimbs of E16.5 (**e**, **f**) and E19 (**g**, **h**) of wild-type (**e**, **g**) and mutant (**f**, **h**) embryos. Reduced ossification is observed in mutant E19 vertebrae (**j**) and P0 supraoccipital bone (**l**) relative to wild-type (**i**, **k**). **B**, Longitudinal sections through the tibia of E18.5 wild-type (**a**) and mutant (**b**) embryos, stained with haematoxylin and eosin. p57^{KIP2} expression was visualized by immunofluorescence (**c**). Collagen X expression was detected in wild-type (**d**) but not in mutant (**e**) hypertrophic chondrocytes; h, humerus; hc, hypertrophic chondrocytes; pc, proliferating chondrocytes; r, radius; rc, reserving chondrocytes; s, scapula; su, supraoccipital bone; u, ulna; x, xiphoid process. Scale bars, 200 μm.

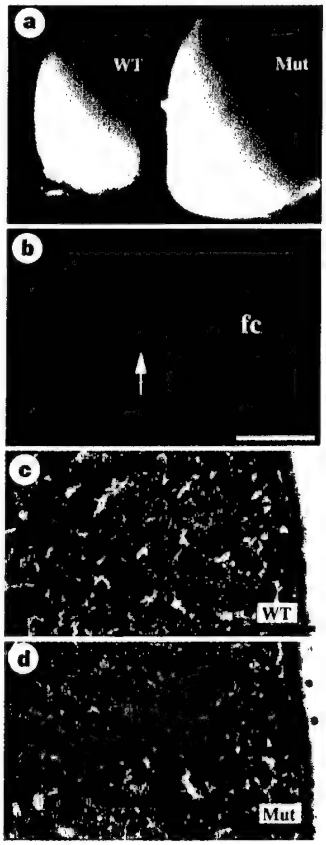

Figure 6 Hyperplasia of the adrenal gland in p57^{KIP2} mutants. **a**, The E19 adrenal gland is enlarged in p57$^{+/-m}$ mice. **b**, p57^{KIP2} protein is expressed in the fetal cortex but not the medulla (arrow) in an E20 adrenal gland. **c**, **d**, Haematoxylin and eosin-stained sections of E20 wild-type (**c**) and p57$^{+/-m}$ (**d**) adrenal glands. Arrows indicate cytomegaly; fc, fetal cortex; m, medulla. Scale bars: **b**, 200 μm; **c**, **d**, 50 μm.

and macroglossia; the mouse tongue has extremely high levels of p57^{KIP2} during development, as does the human placenta, which controls nutrient flow to embryos[10,11].

It is unknown which of the internal manifestations of BWS these two patients exhibit. However, the p57$^{-/-}$ mouse displays several common internal BWS phenotypes including enlargement of the adrenal cortex, adrenal cytomegaly, and renal medullary dysplasia, which are now strongly predicted to be present in the two affected individuals. Phenotypes present in the p57$^{-/-}$ mouse not frequently considered to be associated with BWS are cleft palate, endochondral ossification defects, and ocular lens impairment. It should be noted that, unlike the mouse, human p57^{KIP2} imprinting is incomplete with 5% residual expression from the paternal allele[15]. Therefore, although mice lacking the maternal allele are null mutants, the equivalent humans are hypomorphs. This may affect the relative penetrance of phenotypes between species. Nevertheless, increased frequency of cleft palate[27,28], skeletal anomalies[29], and cataract formation[29] have been reported in conjunction with BWS. A very high frequency of palate defects including cleft and soft palate have been observed in 6 of 10 BWS patients in a study from Japan[28]. The high frequency of palate defects in that study relative to others may reflect the differences in frequencies of modifier genes in distinct populations. In response to this study it was learned that the BWS patient from a previous study[18] who had a maternally inherited null allele in p57^{KIP2} also had a cleft palate (I. Hatada, personal communication). This provides further compelling support for the involvement of p57^{KIP2} in BWS. It has also been observed that the long bones in BWS patients often show widened metaphyses and a thickened bony cortex[21]. This is consistent with our observations of the thickened bones in p57^{KIP2}-deficient mice. Thus the analysis of the p57^{KIP2}-deficient mouse has not only provided proof of the role of p57^{KIP2} in BWS, but has also extended the range of phenotypes expected on further analysis of BWS patients with p57^{KIP2} mutations. Furthermore, it has provided an explanation for the physiological basis of several phenotypes observed in BWS patients, including skeletal abnormalities (chondrocyte proliferation and differentiation), omphalocele (abdominal

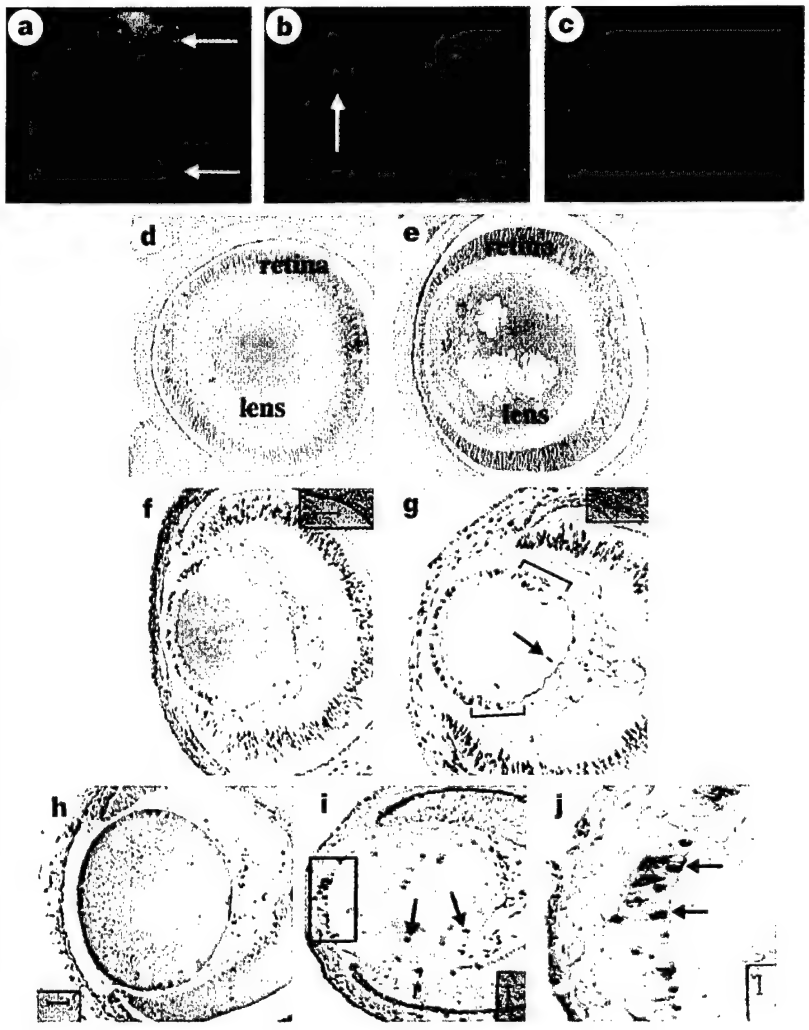

Figure 7 Loss of $p57^{KIP2}$ causes increased proliferation and apoptosis in the lens. **a–c**, Immunofluorescent analysis shows strong $p57^{KIP2}$ expression in all nuclei of the post-mitotic lens fibre cell compartment (arrows) (**a**), occasional nuclear and cytoplasmic staining in anterior epithelial cells (arrow) (**b**), and no expression in $p57^{-/-}$ lenses (**c**). **d**, **e**, Histological presentation of haematoxylin and eosin-stained E15.5 lens sections (sagittal) derived from $p57^{+/+}$ (**d**) or $p57^{-/-}$ (**e**) embryos. **f**, **g**, BrdU incorporation assays on E13.5 lens sections derived from $p57^{+/+}$ (**f**) or $p57^{-/-}$ (**g**) embryos. Brackets show inappropriate S-phase entry in normally postmitotic lens fibre cells of the equatorial region. The arrow indicates S-phase entry in central lens fibre cells. **h–j**, TUNEL assays on E13.5 lens sections derived from $p57^{+/+}$ (**h**), $p57^{-/-}$ (**i**), $p57^{-/-}$ (**j**) embryos (higher magnification of the boxed region in **i** showing abundant apoptotic nuclei in the anterior epithelial layer). Scale bars: **f–i**, 10 μm; **j**, 4 μm.

muscle developmental defects) and adrenomegaly (absence of a cell proliferation inhibitor).

Because $p57^{KIP2}$ is central to BWS, it is likely that most or all BWS forms affect $p57^{KIP2}$ function in some way. Loss of $p57^{KIP2}$ could account for BWS involving alterations at the maternal 11p15.5 locus. However, it is more difficult to imagine how $p57^{KIP2}$ is directly involved in the form of BWS caused by duplication of the paternal 11p15.5 region. It is possible that duplicating the imprinted paternal $p57^{KIP2}$ allele could affect the maternal allele's expression, but this seems unlikely. An alternative explanation is the linked-antagonist model, which proposes that mutations in $p57^{KIP2}$ or overexpression of a $p57^{KIP2}$ antagonist cause BWS. The chromosome specificity of the paternal duplication could be accounted for by exclusive paternal expression of the antagonist, or by the fact that, owing to tight linkage, the duplicated region would also carry $p57^{KIP2}$, and so duplications including the maternal $p57^{KIP2}$ would not produce BWS. Under these conditions, duplication of the paternal 11p15 region would have an effect similar to mutating the $p57^{KIP2}$ gene, increasing a dosage-dependent antagonist. It is possible that IGF2 is the linked antagonist that acts in the same pathway as $p57^{KIP2}$, but in opposition. Increased IGF2 expression leads to some phenotypes associated with BWS in mice[30] and humans[31]. The linked-antagonist model predicts that each mutation (or increased dosage) would have some common effects (for example, omphalocele, macroglossia and gigantism) and some unique effects (for example, earlobe creases, viceromegaly and renal medulary dysplasia), and there would be significant variability in the range of different phenotypes, depending on which genes were mutant or over-produced. Paternal uniparental disomy would cause the most severe phenotype because all $p57^{KIP2}$ expression would be lost and increased dosage of the antagonist(s) would occur. The linked-antagonist model accounts for the highly variable penetrance associated with this syndrome.

Because we now have an understanding of the molecular defects that cause BWS, there should be a concerted effort to correlate phenotype with genotype of BWS patients to establish whether groups of phenotypes are linked and correlate with a particular genotype. This analysis should help establish whether BWS is a single disease or a collection of similar diseases that operate through an overlapping pathway, and will definitively establish whether $p57^{KIP2}$ is a tumour suppressor. □

Methods

Targeting vector construction. The $p57^{KIP2}$ targeting vector was constructed using plasmids containing *PGK-neo* and *pMC1-HSV-tk*. *PGK-neo* on a *Xho*I-*Hin*dIII fragment was cloned into p57Sal1d3 cleaved with *Hin*dIII and partly with *Xho*I to make p57KO-2A, containing a 7-kb fragment of $p57^{KIP2}$ 5′-genomic DNA. A 5.6-kb *Spe*I–*Eco*RV fragment of $p57^{KIP2}$ 3′-genomic DNA was ligated into *Xba*I–*Cla*I (blunt)-digested pMC1TK generating p57KO-2B with *tk* gene 3′ to $p57^{KIP2}$ genomic DNA. To generate the targeting vector p57KO-2, the *Sal*I/*Eco*RV fragment of p57KO-2A containing $p57^{KIP2}$ 5′-genomic DNA and *neo* was ligated into *Sal*1–*Cla*I (blunt)-digested p57KO-2B. p57KO-2 introduces *Eco*RI and *Spe*I sites into $p57^{KIP2}$ locus.

ES cell culture and germline transmission of targeted allele. Linearized p57KO-2 (25 μg) was electroporated into 1.1×10^7 AB2.1 ES cells and cultured[32]. Homologous recombinant clones were microinjected into 3.5-d.p.c.

C57BL/6 blastocysts and a male chimaera derived from clone p57-2.2.12B transmitted the targeted allele.

Gross and histological analysis. Embryos were fixed in 10% formalin or Bouin's. For histological analysis, fixed samples were dehydrated using ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Embedded samples were sectioned at 5 μm.

Immunohistochemistry and *in situ* hybridization. Immunostaining for collagen X was performed as described[23]. For cell proliferation assays, pregnant mice were injected with BrdU (0.1 mg per g body weight) 2 h before caesarean section, and positive cells were identified with an anti-BrdU monoclonal antibody (Dako). *In situ* hybridization was performed as described[5]. Apoptosis and BrdU incorporation assays were performed as described[25].

Protein analysis. Tissues from E18 embryos were lysed in NP-40 buffer and cleared by centrifugation. Protein (30 μg) was immunoblotted with anti-$p57^{KIP2}$ (monoclonal antibody KP90)[33], anti-$p21^{CIP1}$ (monoclonal antibody 65)[6] or anti-$p27^{KIP1}$ antibodies (provided by A. Koff) using ECL detection.

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Genetics

Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, $p57^{KIP2}$, on chromosome 11p15

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ABSTRACT **Parental origin-specific alterations of chromosome 11p15 in human cancer suggest the involvement of one or more maternally expressed imprinted genes involved in embryonal tumor suppression and the cancer-predisposing Beckwith–Wiedemann syndrome (BWS). The gene encoding cyclin-dependent kinase inhibitor $p57^{KIP2}$, whose overexpression causes G_1 phase arrest, was recently cloned and mapped to this band. We find that the $p57^{KIP2}$ gene is imprinted, with preferential expression of the maternal allele. However, the imprint is not absolute, as the paternal allele is also expressed at low levels in most tissues, and at levels comparable to the maternal allele in fetal brain and some embryonal tumors. The biochemical function, chromosomal location, and imprinting of the $p57^{KIP2}$ gene match the properties predicted for a tumor suppressor gene at 11p15.5. However, as the $p57^{KIP2}$ gene is 500 kb centromeric to the gene encoding insulin-like growth factor 2, it is likely to be part of a large domain containing other imprinted genes. Thus, loss of heterozygosity or loss of imprinting might simultaneously affect several genes at this locus that together contribute to tumor and/or growth-suppressing functions that are disrupted in BWS and embryonal tumors.**

Genomic imprinting is a modification of a specific parental chromosome in the gamete or zygote, leading to differential expression of the two alleles of a gene in somatic cells (1). While the strict definition of imprinting does not require monoallelic expression, this is seen for most imprinted genes. A relatively small number of imprinted genes have been identified, although studies of chromosomal uniparental disomy in mice suggest that many more have yet to be identified (2).

Human chromosome 11 was predicted to contain an imprinted, maternally expressed tumor suppressor gene (3), because loss of heterozygosity (LOH) of chromosome 11p15 in Wilms tumor and other embryonal tumors preferentially involves the maternal chromosome (4). Furthermore, balanced chromosomal rearrangements of 11p15 always involve the maternal chromosome in Beckwith–Wiedemann syndrome (BWS) (5), which causes prenatal overgrowth and embryonal tumors such as Wilms tumor (5).

Two imprinted genes have been identified on 11p15. Insulin-like growth factor II gene (*IGF2*), a growth-promoting gene on 11p15, was found to be expressed from the paternal allele (6, 7). Embryonal tumors show loss of imprinting (LOI) and biallelic expression of *IGF2* (6, 7). Closely linked (<100 kb) to *IGF2* is the nontranslated, maternally expressed *H19* gene (6). Mouse embryo knockout experiments show that *H19* imprinting regulates *IGF2* expression, but these animals, while large, do not develop tumors (8). *H19* also lies outside the region of 11p15 that suppresses tumorigenicity in genetic complementation experiments (9). Thus, additional imprinted genes on 11p15 are likely to play a role in tumorigenesis. In addition to the imprinted genes on chromosome 11p15, a number of imprinted genes have been identified over a several megabase region of chromosome 15, including small nuclear ribonucleoprotein N (snRPN), which may be involved in the retardation and obesity-associated Prader–Willi syndrome (10).

$p57^{KIP2}$ is a cyclin-dependent kinase inhibitor that causes G_1 arrest (11, 12) and is homologous in its inhibitory domain to $p21^{CIP1/WAF1}$, a mediator of p53-directed cell cycle arrest (13–15). Since the gene encoding $p57^{KIP2}$ maps to 11p15 (11), we have examined the imprinted state of this gene in normal development and determined if the pattern of imprinting is consistent with a role of $p57^{KIP2}$ gene as a potential tumor-suppressor gene.

MATERIALS AND METHODS

Identification of a Transcribed Polymorphism in $p57^{KIP2}$. PCR amplifications were performed in a final volume of 25 μl of buffer containing 60 mM Tris·HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 60 nM ^{32}P-labeled hR21 (5′-TTCGCGCCCTGCTCGGCGCTCTCTTGAGGC-3′), 400 nM hF19 (5′-TGCCCGCGTTCTACCGCGAGACGGTG-CAGG-3′), 12.5% dimethyl sulfoxide, 0.2 mM each dNTP, 150 ng of genomic DNA, and 1.25 units of exo$^-$ *Pfu* DNA polymerase (Stratagene). Amplification conditions consisted of an initial denaturation of 5 min at 98°C, followed by 35 cycles of 1 min at 98°C, 1 min at 65°C, and 2 min at 75°C. A final extension of 10 min was carried out at 75°C. After PCR, the amplified fragment was digested with *Pvu* II and then applied to a 4% acrylamide sequencing gel. Allele sizes were determined by comparing migration relative to an M13 sequencing ladder. For sequence analysis, the PCR fragment amplified by using 400 nM each of hF19 and hR21 under the same conditions was cloned into the *Sma* I site in pBluescript (Stratagene). Multiple isolates were sequenced to eliminate PCR artifacts.

DNA and RNA Preparation from Tissue Samples. Wilms tumors and corresponding normal kidneys were obtained from snap-frozen surgical specimens, and fetal tissue samples were obtained from the University of Washington Fetal Tissue Bank.

DNA was extracted from tissue samples by proteinase K/SDS treatment. Pea-sized tissue fragments were pulverized in liquid nitrogen and resuspended in 5 ml of TE9 (500 mM

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Abbreviations: LOH, loss of heterozygosity; LOI, loss of imprinting; BWS, Beckwith–Wiedemann syndrome; RT, reverse transcription; *IGF2*, gene for insulin-like growth factor II.

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Tris/20 mM EDTA/10 mM NaCl, pH 9.0). One hundred microliters of proteinase K at 10 mg/ml and 250 μl of 20% SDS were added per sample, followed by incubation overnight at 55°C. Samples were extracted twice with phenol/chloroform and once with chloroform. DNA was precipitated by the addition of 0.3 volumes of 10 M NH_4OAc and 2.5 volumes of ethanol. DNA pellets were washed with 70% ethanol, air-dried, and resuspended in 100–500 μl of TE (3 mM Tris/0.2 mM EDTA, pH 7.5). For RNA extraction, tissue samples were diced with a sterile scalpel, and RNA was isolated by using the MicroFastTrack mRNA isolation kit (Invitrogen) and resuspended in 50 μl of the buffer provided.

Characterization of $p57^{KIP2}$ PAPA Polymorphism in Tissue Samples. DNA isolated from human tissue samples identified individuals heterozygous for a transcribed polymorphism which involves a hexanucleotide repeat within exon 1 of the $p57^{KIP2}$ gene encoding a proline-alanine repeat called the PAPA repeat. Primers hF19 and hRT1 (5′-CGCTGATCTCT-TGCGCTTGGCGAAGAAATC) were used for PCR amplification of the hexanucleotides encoding the PAPA repeat domain of $p57^{KIP2}$. PCR reactions were performed in a final volume of 50 μl of buffer A (Stratagene) containing 100 ng of each primer, 0.25 mM each dNTP, 10% dimethyl sulfoxide (DMSO), and 1–2 units of *Pfu* exo^+ DNA polymerase (Stratagene). PCR reactions were carried out as follows with the Perkin–Elmer/Cetus 9600 PCR thermal cycler: 98°C for 5 min; 35 cycles of 98°C for 1 min, 60°C for 1 min, and 72°C for 2 min (35 cycles); and an extension at 72°C for 10 min. PCR products were digested with *Pvu* II and electrophoresed on a 6% nondenaturing polyacrylamide gel to resolve the polymorphic size difference in the PAPA repeat region.

Reverse Transcription (RT)–PCR Reactions for Analyzing Allele-Specific Expression. RT-PCRs were done with mRNA purified from informative (heterozygous) tissues as described above. mRNA (1–5 μl) was initially treated with DNase to remove contaminating DNA in a final volume of 30 μl of *Taq* PCR buffer (Boehringer Mannheim) containing 10 units of DNase and 0.5 units of RNase Inhibitor (5′-3′). DNase treatment was carried out at 37°C for 60 min, followed by inactivation of the enzyme at 95°C for 7 min.

DNase-treated mRNA was cooled to 65°C, followed by addition of the primer hRT1 (final concentration, 5 ng/μl) and incubation for 5 min. Reactions were then cooled to 53°C, followed by addition of stock RT solution (1 μl of ×10 *Taq* PCR buffer/8 μl of dNTPs at 1.25 mM each) and 1 μl of avian myeloblastosis virus (AMV) reverse transcriptase (Seikagaku America, Rockville, MD; 30 units/μl). RT reactions were carried out at 53°C for 60 min, followed by heat inactivation at 95°C for 7 min. RT reactions were purified by using Qiagen (Chatsworth, CA) Qiaquick PCR purification columns per kit instructions. RT products were eluted in 40 μl of 10 mM Tris (pH 8.3). Twenty microliters of the purified reaction mixture was then used in the PAPA repeat PCR reaction with hF19 and hRT1 as the primers.

RT-PCRs were initially analyzed by electrophoresing 5–10 μl of the 50-μl PCR reaction mixture on a 1% agarose gel to ensure that no contaminating genomic DNA had been amplified during the PCR. When no contamination was present, 4–17 μl of the PCR reaction was digested with *Pvu* II and analyzed on a 6% nondenaturing polyacrylamide gel. Corresponding PCRs of genomic DNA from the same tissue were run simultaneously to assay allele-specific expression. Gels were transferred to Genescreen (NEN/DuPont) by electroblotting with a Trans-Blot Cell apparatus (Bio-Rad). Blots were probed with an [α-^{32}P]dATP-labeled probe spanning the PAPA repeat region. Digital quantification of expression levels was done with a PhosphorImager (Molecular Dynamics).

RESULTS

Identification of a Transcribed Polymorphism in $p57^{KIP2}$. Sequence analysis of human $p57^{KIP2}$ gene revealed a hexanucleotide repeat within exon 1 encoding a proline–alanine repeat termed "PAPA" repeat (11). PCR amplification and sequencing of this domain from 60 individuals revealed two common and two rare alleles of a length polymorphism in this repeat (Fig. 1). Analysis of reference kindreds showed Mendelian segregation of these alleles (Fig. 1). Sequence analysis revealed that the polymorphism represented deletion of two to eight codons in the PAPA domain (Fig. 1).

Imprinting of the $p57^{KIP2}$ Gene in Developing Tissues. To determine whether one or both alleles were expressed in heterozygous individuals, RT-PCR was performed with primers that spanned the PAPA repeat and first intron (Fig. 2). Conditions were developed to isolate the PCR-amplified cDNA product to examine allele-specific expression. Use of primers spanning the first intron allowed for distinction be-

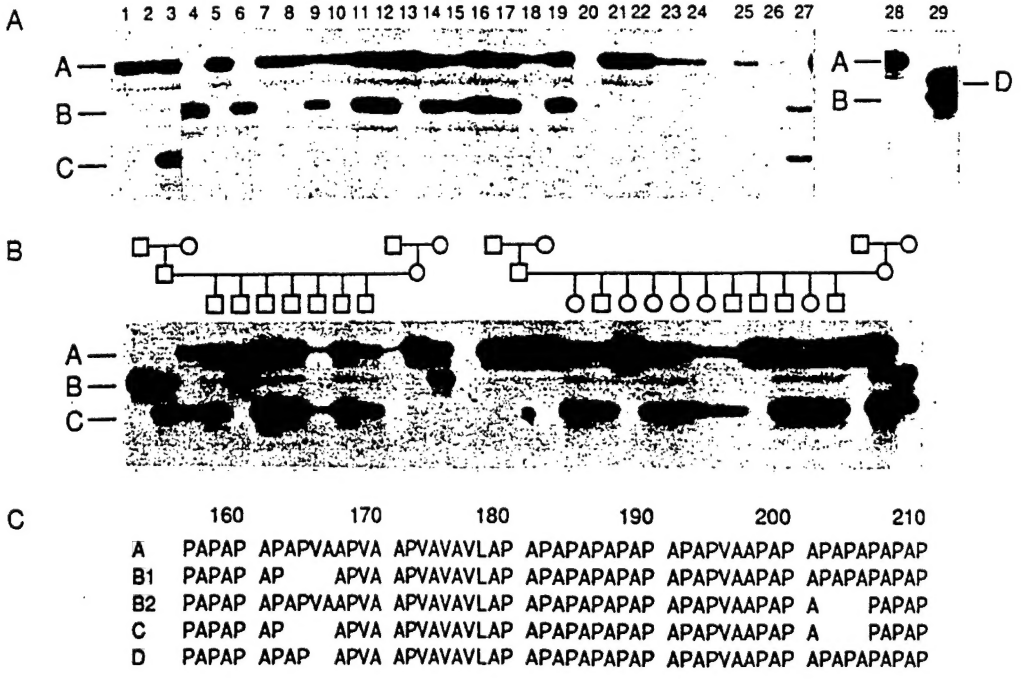

FIG. 1. Polymorphic nature of the PAPA repeat region of human $p57^{KIP2}$. (*A*) PCR analysis of the PAPA repeat region in a human population. Samples in lanes marked 1–27 were derived from Centre d'Etude du Polymorphisme Humain (CEPH) families. Samples 28 and 29 are from non-CEPH normal individuals. (*B*) PCR analysis of the PAPA repeat region in a normal family. The pedigrees shown represent CEPH families. (*C*) Amino acid sequence variations of the PAPA repeat region observed in the polymorphisms shown in *A*.

tween genomic amplification versus cDNA amplification; thus, genomic DNA contamination in the RT-PCRs could be easily ruled out.

DNA from 17 fetuses was analyzed for the $p57^{KIP2}$ polymorphism, and 6 heterozygotes were identified. All 6 showed preferential expression of a single allele in most tissues, suggesting that the gene is imprinted in normal human development (Fig. 3, Table 1). In one case, maternal DNA from the decidua allowed assignment of the preferentially expressed allele as being of maternal origin.

The one exceptional tissue was brain, which showed comparable expression of the two alleles in both informative cases for which this tissue was available. The difference in expression levels of the two alleles in brain was ≈2-fold. Other tissues also showed some degree of expression of the other (presumably paternal) allele, although the degree of expression of that allele was much lower than seen in brain (Fig. 3, Table 1) and in many samples was virtually undetectable. Low-level expression of the (presumed) paternal allele was ≈1/20th of that of the corresponding maternal allele in all other nonbrain tissues, which was confirmed by reconstitution experiments at various dilutions of the two alleles (J.S.T., unpublished data).

To determine conclusively the parental origin of the expressed $p57^{KIP2}$ allele, normal kidneys from Wilms tumor patients were examined, and, when possible, parental DNA was obtained from blood samples. From 53 Wilms tumor patients (and one hepatoblastoma patient), 15 informative normal kidneys were obtained for RT-PCR analysis. In all 15 samples, preferential expression of one $p57^{KIP2}$ allele was observed, as found in the fetal samples (Fig. 4). Low-level expression of the paternal allele was observed in some normal kidney samples; however, the expression level of the maternal allele exceeded that of the paternal allele by an average ratio of 9:1. Of these 15 samples, parental identity of the expressed allele was determined directly from parental DNA of five of the patients (Fig. 4 *A* and *B*; Table 1), which verified maternal expression of $p57^{KIP2}$. Maternal expression of the $p57^{KIP2}$ gene was determined for an additional sample on the basis of flanking markers on 11p15 (patient 19, Table 1), and it was inferred in the case of four other patients on the basis of presumed maternal loss of heterozygosity in the corresponding tumor (indicated in the legend to Table 1). Thus, maternal-specific expression of $p57^{KIP2}$ gene was verified in 11 different normal individuals (7 direct, 4 inferred; 10 postnatal kidneys, 1 fetal specimen).

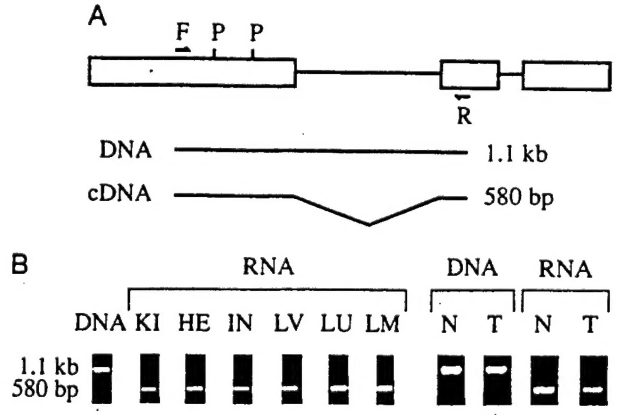

FIG. 2. Amplification of the PAPA repeat polymorphism. (*A*) Schematic representation of the $p57^{KIP2}$ gene. Boxes indicate the three exons, with single lines between the boxes indicating the introns. F and R indicate primers hF19 and hRT1, respectively, utilized for both typing of tissue samples and for RT-PCR reactions. P indicates *Pvu* II sites used for digestion of PCR products as described in *Materials and Methods*. Below the gene is a map indicating the relative sizes of PCR products generated with either a genomic or a reverse-transcribed RNA template. (*B*) PCR products from either genomic DNA or reverse-transcribed RNA (cDNA), isolated from fetal tissues (first seven lanes) or from a tumor/normal kidney pair (last four lanes). KI, kidney; HE, heart; IN, intestine; LV, liver; LU, lung; LM, limb; N, normal kidney; T, Wilms tumor. Note that lack of genomic-sized PCR products in the cDNA lanes indicates that no genomic DNA contamination was present in the RT-PCRs.

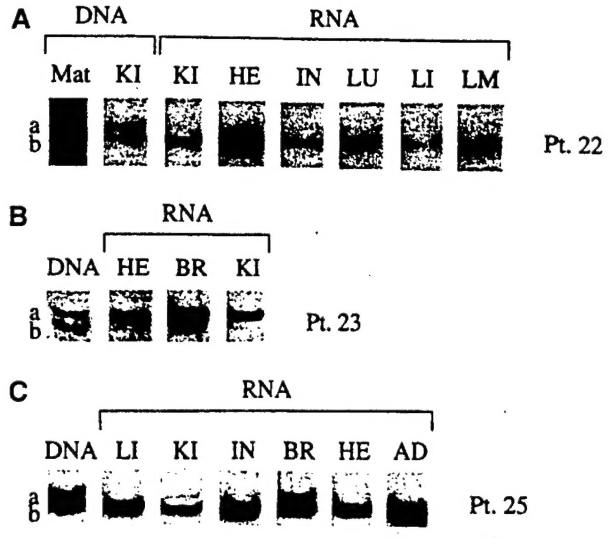

FIG. 3. RT-PCR analysis of expression of $p57^{KIP2}$ from fetal tissue samples. mRNA samples isolated from heterozygous fetal tissue samples were analyzed by RT-PCR as described in *Materials and Methods* and as outlined in Fig. 2. Each set includes PCR of genomic DNA isolated from fetal tissues, along with RT-PCRs of RNA from the indicated tissues. KI, kidney; HE, heart; IN, intestine; LU, lung; LI, liver; LM, limb; BR, brain; AD, adrenal. A and B alleles are shown on the left of each panel. (*A*) Patient 22 (Table 1). Mat refers to maternal DNA isolated from accompanying decidua. (*B*) Patient 23 (Table 1). (*C*) Patient 25 (Table 1).

Imprinting of $p57^{KIP2}$ Gene in Wilms Tumors. Alteration of imprinting patterns has been observed previously in embryonal tumors. Specifically, the maternal copy of *IGF2* and *H19* have been found to undergo a conversion to a paternal

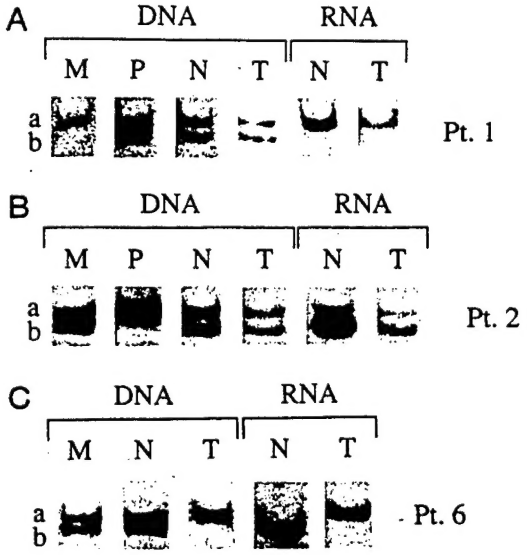

FIG. 4. RT-PCR analysis of expression of $p57^{KIP2}$ from Wilms tumors and corresponding normal kidneys. mRNA from tumor and normal kidney was analyzed as described in Figs. 2 and 3. PCR of maternal (M) and paternal (P) DNA isolated from blood, DNA from normal kidney (N) and tumors (T), and RT-PCR reactions (labeled RNA) are indicated. A and B alleles are shown on the left of each panel. (*A*) Patient 1 (Table 1; non-LOH). (*B*) Patient 2 (non-LOH). (*C*) Patient 6 (LOH).

Table 1. Allele-specific expression of $p57^{KIP2}$ in normal tissues and embryonal tumors

Patient	Tissue	DNA	RNA	Patient	Tissue	DNA	RNA
1	Normal kidney	A/B	$A_{mat}/-$	19	Normal kidney	A/B	$A_{mat}/-$
	Wilms tumor	A/B	$A_{mat}/-$	20	Normal kidney	A/D	$A_{mat}/-$
2	Normal kidney	A/B	$-/B_{mat}$		Wilms tumor	A/D	$A_{mat}/-$
	Wilms tumor	A/B	$-/B_{mat}$	21	Normal liver	A/B	A/−
3	Normal kidney	A/B			Hepatoblastoma	A/B	A/−
	Wilms tumor	A/B	$A_{mat}/-$	22	Fetal kidney	A/B	$-/B_{mat}$
4	Normal kidney	A/C			Fetal heart	A/B	$-/B_{mat}$
	Wilms tumor	A/C	A/C		Fetal intestine	A/B	$-/B_{mat}$
5	Normal kidney	A/B			Fetal lung	A/B	$-/B_{mat}$
	Wilms tumor	−/B	−/B		Fetal liver	A/B	$-/B_{mat}$
6	Normal kidney	A/B	−/B		Fetal limb	A/B	$-/B_{mat}$
	Wilms tumor	A/−	A/−	23	Fetal heart	A/B	A/−
7	Normal kidney	A/B	−/B		Fetal brain	A/B	A/B
	Wilms tumor	A/B	−/B		Fetal kidney	A/B	A/−
8	Normal kidney	A/B	−/B	24	Fetal lung	D/B	−/B
	Wilms tumor	A/−	A/−		Fetal gut	D/B	−/B
9	Normal kidney	A/B	A/−		Fetal kidney	D/B	−/B
	Wilms tumor	A/B	A/−		Fetal adrenal	D/B	−/B
10	Normal kidney	A/B	−/B		Fetal liver	D/B	−/B
	Wilms tumor	A/B	−/B		Fetal heart	D/B	−/B
11	Normal kidney	A/B		25	Fetal liver	A/B	−/B
	Wilms tumor	A/B	$A_{mat}/-$		Fetal kidney	A/B	−/B
12	Normal kidney	A/B			Fetal intestine	A/B	−/B
	Wilms tumor	A/B	−/B		Fetal brain	A/B	A/B
13	Normal kidney	A/B			Fetal heart	A/B	−/B
	Wilms tumor	A/−	A/−		Fetal adrenal	A/B	−/B
14	Normal kidney	A/B	−/B	26	Fetal lung	A/B	−/B
	Wilms tumor	A/B	A/B		Fetal spinal cord	A/B	−/B
15	Normal kidney	A/B	$-/B_{mat}$		Fetal intestine	A/B	−/B
	Wilms tumor	A/−	$A_{pat}/-$		Fetal liver	A/B	−/B
16	Normal kidney	A/B	$A_{mat}/-$		Fetal kidney	A/B	−/B
	Wilms tumor	A/B	$A_{mat}/-$	27	Fetal intestine	A/B	−/B
17	Normal kidney	A/B	−/B		Fetal kidney	A/B	−/B
	Wilms tumor	A/−	A/−		Fetal heart	A/B	−/B
18	Normal kidney	A/B	−/B		Fetal adrenal	A/B	−/B
					Fetal ovary	A/B	−/B

Analysis of $p57^{KIP2}$ expression from informative individuals based on the PAPA repeat polymorphism. Relevant tissue is indicated in the second column. $p57^{KIP2}$ alleles determined from DNA typing are indicated in the third column. A dash indicates loss of heterozygosity in the tumor. Expression of alleles from RT-PCR analysis is indicated in the column marked RNA. In the cases where parental identity of the alleles could be determined on the basis of parental DNA informativity, the term "mat" is marked next to the expressed allele to designate it as of maternal origin, or "pat" to designate paternal origin. Parental identity of alleles from patients 1, 2, 3, 11, 15, 16, and 22 were determined directly from informativity of the $p57^{KIP2}$ PAPA polymorphism from parental DNA. Parental identity of patient 19 was determined by informativity of flanking polymorphic markers on 11p15. All other LOH tumors without designations are assumed to be maternal LOH.

epigenotype, resulting in biallelic expression of *IGF2* and loss of expression of *H19* (16, 17). Given these observed changes in tumors and the potential connection between $p57^{KIP2}$ and cancer, we examined both non-LOH and LOH Wilms tumors to determine if imprinting of $p57^{KIP2}$ gene was altered.

Twelve non-LOH Wilms tumors informative for $p57^{KIP2}$ were identified and examined for the imprinting status of $p57^{KIP2}$ gene. As found for the normal kidney samples, monoallelic expression was observed in nearly all tumors, with maternal identity verified in six of the tumors (Fig. 4 *A* and *B*; Table 1). Some expression of the paternal allele was also observed in these tumors, with an average maternal/paternal expression ratio of 6:1 (comparable to the corresponding normal kidneys). Of the 12 tumors, 10 displayed a normal pattern of imprinting compared to normal kidney, while 2 of the tumors showed nearly equal biallelic expression of $p57^{KIP2}$, with a <2-fold difference in expression between the two alleles, suggesting LOI. While LOI of *IGF2* and *H19* is common in Wilms tumors (≈70%), LOI of $p57^{KIP2}$ appears to be a relatively rare event. One non-LOH hepatoblastoma was also examined, and normal imprinting was observed.

A prediction of the imprinted tumor-suppressor gene model is that LOH, which preferentially affects the maternal allele, would result in loss of expression of the gene (3). However, since $p57^{KIP2}$ gene is incompletely silenced by imprinting, we did not expect to see complete loss of expression in maternal LOH tumors. In six Wilms tumors informative for the $p57^{KIP2}$ polymorphism and showing LOH, preferential expression of the maternal allele was observed in the normal tissue, but maternal allele expression was absent in the tumor samples (Fig. 4*C*). However, expression of the paternal allele was observed in these tumors, indicating, as expected, that loss of the normally expressing maternal allele does not result in complete loss of $p57^{KIP2}$ expression. Since the RT-PCR assay employed was not quantitative, we are not able to draw a conclusion concerning the level of expression from the paternal chromosome.

DISCUSSION

In summary, the $p57^{KIP2}$ gene is imprinted in humans, the first cell cycle-regulatory gene found to be regulated by this mechanism. While the maternal allele shows preferential expression, the paternal allele is also expressed at low levels in most tissues, but at levels comparable to the maternal allele in developing brain and some embryonal tumors. Low-level

expression of the paternal allele may reflect incomplete repression of that copy, although conceivably a small subpopulation of cells within a tissue may not be imprinted.

The identification of imprinting of a cyclin-dependent kinase inhibitor is a surprising result. If $p57^{KIP2}$ is a bona fide tumor suppressor or growth regulator, monoallelic expression would seem to increase the risk of developmental malformation and cancer. Perhaps $p57^{KIP2}$ is most important during embryogenesis, thereby making the temporal window for mutation small. It may contribute only marginally to maintenance of the nonproliferative state in adult tissues and thus not be a significant suppressor of growth. Alternatively, it is possible that its levels are tightly controlled and that the weak expression of the paternal allele may be enhanced when the maternal allele is lost in adult tissues, allowing it to escape the effects of imprinting to some degree. These experiments will be best addressed when mice lacking $p57^{KIP2}$ are available. The parental specificity of the imprint, with expression of the maternal allele of $p57^{KIP2}$, is consistent with the general paradigm that imprinted genes expressed from the maternal allele tend to inhibit cell growth and may have arisen to mediate maternal–fetal conflicts for nutrients, as proposed by Haig and Graham (18).

The localization of the $p57^{KIP2}$ gene to 11p15, its maternal-specific expression, and its biochemical potential for growth regulation match the properties predicted for a tumor suppressor involved in embryonal tumors and BWS. We have found that the $p57^{KIP2}$ gene lies within 100 kb of a cluster of five maternal balanced chromosomal rearrangement breakpoints from BWS patients (19), and its expression in BWS might be influenced by these breakpoints. It is also of interest that p16, another cyclin-dependent kinase inhibitor involved in some familial melanomas (20), shows decreased expression and increased methylation in some tumors in which it is not mutated (21). Thus, it will be important to determine whether other cdk inhibitors are also imprinted.

Third, these results, combined with our localization of $p57^{KIP2}$ gene on an 11p15 cosmid contig (19), indicate that there is a 450- to 500-kb domain extending from $p57^{KIP2}$ gene to *H19*, containing multiple imprinted genes. This is supported not only by the relative proximity of these genes, but also by the fact that *IGF2* and *H19*, like $p57^{KIP2}$ gene, are not imprinted in some brain tissues (22). Not all genes within this domain are necessarily imprinted, as observed in the Prader–Willi syndrome region of 15q11-12 (23), but it seems likely that other imprinted genes within this domain will be identified that may contribute to embryonal tumors and BWS.

Finally, these data suggest that establishment of an imprint may be coordinately regulated throughout the entire domain, as suggested by similar tissue-specific expression and imprinting patterns of *IGF2*, *H19*, and $p57^{KIP2}$ gene (ref. 11 and this report), while loss of imprinting may not necessarily affect the entire region. LOI of *IGF2/H19* involves an epigenetic switch of the maternal allele to a paternal epigenotype (16). We find that LOI of *IGF2/H19* is not necessarily linked to the LOI of the $p57^{KIP2}$ gene [patients 1, 2, and 11 show a LOI of *IGF2* (6, 16) but not of the $p57^{KIP2}$ gene]. Thus, while imprint establishment may occur across 11p15 by a common mechanism (analogous to X-chromosome inactivation), LOI may reflect a breakdown in imprint maintenance, which may be regulated independently for each gene.

Note Added in Proof. After submission of this manuscript, imprinting of $p57^{KIP2}$ in mouse was reported (24).

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